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Shrimp Production Environment and the Gut Microbiome: Effects of Aquaculture Practices and Selective Breeding on the Gut Microbiome of Pacific Whiteleg Shrimp, *Litopenaeus vannamei*

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SHRIMP PRODUCTION ENVIRONMENT AND THE GUT MICROBIOME: EFFECTS OF AQUACULTURE PRACTICES AND SELECTIVE BREEDING ON THE GUT MICROBIOME OF PACIFIC WHITELEG SHRIMP, *LITOPENAEUS VANNAMEI*

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This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science in Biological Sciences degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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This thesis is in dedication to:

My parents: Steven & Vicki Hansen

who impressed upon me the value of education, setting goals and to never stop learning

My loving husband: Heath Landsman

my best friend who continually offered his support and encouragement with a good dose of reality

My two curious boys: Devin & Kaden

who inspire me daily to seek not only answers but also new questions

My brother: Nicholas Hansen

who taught me so many things about optimism, persistence and overcoming preconceived ideas and obstacles

My sister: Tonja Cantú, Ph.D.

who instilled a love of learning in me at a young age and never stopped learning herself

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ABSTRACT

SHRIMP PRODUCTION ENVIRONMENT AND THE GUT MICROBIOME: EFFECTS OF AQUACULTURE PRACTICES AND SELECTIVE BREEDING ON THE GUT MICROBIOME OF PACIFIC WHITELEG SHRIMP, *LITOPENAEUS VANNAMEI*

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Knowledge of the functional role of the gut microbiome in animal health and nutrition may provide solutions to shrimp aquaculture challenges, such as improving disease resistance and optimizing growth particularly with low cost feeds. Successful manipulation of bacteria found in the gut requires a deeper understanding of shrimp microbial communities and how their compositional structure is influenced by environmental conditions, and inherent host factors such as genetics.

The initial research investigated the intestinal bacterial communities of the Pacific whiteleg shrimp (*Litopenaeus vannamei)* reared in pond systems compared to indoor aquaculture facilities as an exploration of the effects of aquaculture practices on the acquired gut microbiome. Ponds averaged a depth of 1.5 meters, with stocking densities maintained within a range typical of intensive production systems (30–60 shrimp/m^3). Water chemistry testing was conducted weekly to monitor levels of TAN, nitrite, nitrate and alkalinity. These parameters were used to determine the rates of water exchange to maintain water quality, which ranged from 0% to 20%. Feed was offered at scheduled times during the day (5 a.m., 12 p.m. and 5 p.m.). Indoor production shrimp were maintained at 28 ± 1 °C in temperature-controlled tanks. Water management was carried

out using separate recirculating aquaculture systems, one for each tank, utilizing fresh water processed by reverse osmosis, and then mixed with 28 g Marinemix (Marine Enterprises International, LLC., Baltimore, MD, USA) per liter of production water. Total ammonia nitrogen (TAN) levels were maintained at less than 3.0 mg/mL ($NH_3 \leq$ 0.2), nitrite levels below 4.5 mg/mL, and nitrate levels never exceeded 100 mg/mL. Evaporated water was replaced with fresh water as needed to maintain salinity at 28 parts per thousand (ppt). Intestinal bacterial community profiles were different between each production system. Bacteria affiliated with Rhodobacteraceae (Proteobacteria) and Actinobacteria were significantly more abundant in indoor cultured shrimp (84.4% vs. 5.1%; 3.0% vs. 0.06%, respectively), while Vibrionaceae (Proteobacteria) (0.03% vs. 44.8%), Firmicutes (0.7% vs. 36.0%), Fusobacteria (0.0% vs. 7.9%), and Cyanobacteria (0.001% vs. 1.6%) were predominant in pond raised shrimp. The results indicate that aquaculture practices greatly influence the intestinal bacterial profile of whiteleg shrimp, and further suggest the bacterial communities of this economically important crustacean could be effectively manipulated using diet composition or environmental factors such as water chemistries.

A subsequent research consisted of two experiments focused on two genetic families of Pacific whiteleg shrimp. One family was selected for specific pathogen resistance (Shrimp Improvement Systems, SIS), while the other genetic line was selected for growth (Oceanic Institute, OI). Both stocks of postlarvae juvenile shrimp were reared in a biosecure / indoor aquaculture facility under the same practices described in the prior study. Two genetic lines of *Litopenaeus vannamei* were used: Shrimp Improvement Systems (SIS, Islamorada, Florida, USA), selected for disease resistance, and Oceanic

Institute (OI, Oahu, Hawaii, USA), selected for growth. During each trial, three replicate tanks were supplemented with a commercial probiotic, while the remaining three tanks did not receive any supplementation (controls). Stocking densities were maintained in the standard range of intensive production systems at 30–60 shrimp per cubic meter, with feed offered continuously. Production tanks (2,921 L) were maintained for 72 days with six tanks for each genetic family, three replicate tanks receiving probiotic infusions and three without. A commercial product, BioWish 3P, was obtained from BioWish Technologies (Cincinnati, Ohio). BioWish 3P contains *Pediococcus acidilactici* $\geq 1 \times 10^8$ cfu/g, *Pediococcus pentosaceus* $\geq 1 \times 10^8$ cfu/g, *Lactobacillus plantarum* $\geq 1 \times 10^8$ cfu/g, *Bacillus subtilis* $\geq 1 \times 10^7$ cfu/g. The freeze-dried bacterial cultures are delivered in an inert carrier, cereal food fines. BioWish 3P was added a rate of 0.73 ± 0.05 g per trial tank daily and was dosed with the feed offered over 24h. Microbiome sampling occurred on day 43 (prior to probiotic additions), day 57, and day 71 (28 days following initial probiotic introduction). Water management was maintained at 28 ± 1 °C and 28 ppt salinity utilizing artificial seawater. Total ammonia nitrogen, nitrite, and nitrate levels were maintained at less than 3.0 mg/mL ($NH_3 \leq 0.2$), below 4.5 mg/mL, below 100 mg/mL respectively.

Considering that the gut microbiome of shrimp begins to develop immediately after hatching and is dependent on the initial diets and environmental conditions, it was not surprising to discover the initial phylum abundance between groups on day 43 differed, with a range of 72.3- 82.5% being Proteobacteria in the SIS shrimp compared to 61.1-65.1% in OI shrimp. The two genetic lines of shrimp revealed significant differences in gut microbiome by family and sampling time points following the

inclusion of probiotics into the diet. After 28 days of treatment on tenure day 71, significant variation became evident in the second most abundant phylum in the SIS shrimp with Bacteroidetes increasing from 4.0% on day 43 to 8.6% on day 71 with probiotics, and from 5.6% on day 43 to 30.6% on day 71 without probiotics. By comparison, the second most abundant phylum in the OI shrimp was Firmicutes, increasing from 0.5% on day 43 to 22.1% on day 71 with probiotics, and from 2.8% to 36.7% without probiotics. As *Pediococcus*, *Lactobacillus,* and *Bacillus*-affiliated OTUs were found in only very low abundance or were undetectable in the gut of probiotic supplemented shrimp, bacterial species from the commercial probiotic formulation did not appear to efficiently colonize the shrimp gut in this study. However, their presence or absence did impact the development of gut bacterial communities through abundances. While future investigations will be necessary to uncover the mechanisms involved, it could be hypothesized that, even if probiotic bacterial species do not become established in high density in the gut of exposed shrimp, they produce metabolites that favor the establishment of certain bacterial species or OTUs over others.

These results suggest that bacterial communities of this economically important crustacean can be effectively manipulated utilizing environmental conditions. They further indicate that development of direct fed microbial strategies to effectively manipulate the microbiome of this important seafood will likely need to take into serious consideration the genetic background of the shrimp genetic lines used in aquaculture production.

INTRODUCTION

Over the last several decades many attempts have been made, and most have failed, to develop and manage biosecure / indoor shrimp production systems that are more profitable than outdoor pond systems (Jobling, 2013). Indoor systems are capital intensive and must be well managed for water chemistry and biology (Alday-Sanz, 2010). Densities required to turn a profit can cause stress to the shrimp, and under suboptimal conditions often lead to reduced growth and eventually death (Alday-Sanz, 2010). These operational requirements come at a cost unique to indoor aquaculture.

Genetic selection of shrimp for rapid growth, while promoting health through microbial management, will likely be critical to insure the success of indoor aquaculture production (Jobling, 2013). Genetic lines of shrimp have been developed for either growth or disease resistance, as the two have been shown to be mutually exclusive in inheritance (Andriantahina, Liu, Feng, & Xiang, 2013). Species of shrimp used in both indoor and outdoor production are the same. Breeding programs focus on disease resistance for outdoor systems, as they have higher exposure to opportunistic pathogens (Andriantahina et al., 2013). Meanwhile, genetic lines developed for biosecure, indoor systems have focused on growth, due to the inherent nature of a low to no pathogen environment (Andriantahina et al., 2013). Use of rapidly growing shrimp, with limited disease resistance, can become problematic when systemic stressors promote growth of ubiquitous and potentially pathogenic species such as *Pseudomonas* and *Vibrio* (E. Li et al., 2018). Even at very low concentrations these bacteria colonize the shrimp gut and environment to cause shrimp disease (E. Li et al., 2018).

Historically, probiotics have been used in pond systems to provide competitive exclusion of pathogens during stress events (Samocha et al., 2004). In biosecure indoor systems, probiotics may also serve the purpose of shifting in the digestive microbiome, allowing the shrimp to achieve improved health and digestive function. Initial contributors to the microbiome come from live feed diets of juvenile shrimp (E. Li et al., 2018). Over time, the digestive microbiome shifts and adapts as the food source changes.

Aquaculture feeds are progressively shifting to contain nutritional sources not found in wild shrimp diets, such as plant proteins, fiber, and carbohydrates (Huang, Li, Wang, & Shao, 2016). Moreover, feeds are often treated to reduce or eliminate microbial contaminants, and thus beneficial microbiome development may be altered (Huang et al., 2016). Determining the differences in the digestive microbiome of various genetic lines, grown in the presence or absence of probiotics, may establish methods to improve health, disease resistance, and growth through gut microbiome development.

To test this hypothesis, six indoor commercial production tanks were utilized for two feeding trials. The initial trial utilized shrimp selected for disease resistance, sourced from Shrimp Improvement Systems in Florida, USA. A common feed, formulated using plant proteins non-native to the shrimp's natural diet, was offered to all tanks. Three of the tanks were treated with a commercial blend of probiotics formulated for use in shrimp production. The remaining three tanks received no probiotic treatments. The microbial ecology of the water columns in these tanks was monitored weekly to quantify total heterotrophic bacterial and the ratio of pathogenic to non-pathogenic *Vibrio* species. Water quality was maintained at a total ammonia nitrogen (TAN) at or below 3.0 mg/L ($NH_3 \leq 0.2$ mg/L), nitrite at or below 4.5 mg/L, pH between 7.3 and 8.0 and alkalinity at

or above 180 mg/L. Each tank was monitored regularly and adjusted individually by adding sodium carbonate to elevate pH and/or sodium bicarbonate to elevate alkalinity as needed. TAN and nitrite never exceeded maximum levels and therefore required no corrective action. To determine if probiotic additions led to improved growth performance and health, shrimp weights and health status were evaluated weekly. Gut samples were also collected and analyzed by 16S sequencing to determine if probiotic microbes established in the gut. Finally, tissue proximate analysis was performed on the shrimp prior to and following the treatment period. The second trial utilized shrimp selected for growth performance, sourced from Oceanic Institute in Hawaii, USA. Otherwise all conditions were identical to the first trial.

Water quality, microbial ecology, and shrimp performance data were analyzed to determine if probiotic treatment improved health, disease resistance, and growth through gut microbiome development. Microbiome profiles were compared to determine if the differing operational methods contributed to altered performance, while the proximate data allowed for observation of nutritional variances possibly linked to enhanced diet utilization. The assembling of all data was used to make a recommendation for future operations and suggestions for future research.

LITERATURE REVIEW

1. **Industry Overview**

There are nearly 3,000 known species of shrimp in the world (Greenberg, 2012). Of those species, less than 3.4% are economically significant. Annual catch reports show roughly 100 species are commonly caught, but 83% of that catch is comprised of only 6 species (Gillett, 2008). The top 6 species of value for fishing are derived from temperate water habitats; areas not experiencing extremes in terms of precipitation or temperature. These species include: *Acetes japonicas,* also known as Akiami Paste shrimp; *Pandalus borealis,* commonly known as Pink shrimp; *Litopenaeus setiferus,* also known as Northern White shrimp; *Macrobrachium rosenbergii,* the Giant River prawn; *Penaeus monodon,* known as Giant Tiger prawn; and *Crangon crangon,* commonly referred to as Sand shrimp (Gillett, 2008; Leung, 2006).

Although the shrimp fishery is one of the most important internationally traded, producing more than 3.4 million tons each year, the wild harvest catch has grown stagnant (Gillett, 2008). Demand for shrimp per capita is stable, but the world's population continues to grow. It is estimated that the world's shrimp supply will have to double in the next 20 years to meet demand (Jobling, 2013). The best alternative to meet this demand is to employ aquaculture (Jobling, 2013). In 2018 it was estimated that 55% of annual global shrimp supply was a produced via aquaculture (Gaille, 2018). Current production is strongly focused on two species *Litopenaeus vannamei* (White leg shrimp) and *Penaeus monodon* (Giant Tiger prawn) (Leung, 2006).

The market price for shrimp is highly dependent on the aquaculture industry. With wild shrimp catch representing a steady, and at times declining balance in the global

market over the past 30 years it is not surprising to discover 42% of the total world shrimp sales come from *Litopenaeus vannamei* farming (Mayes, 2018). When shrimp aquaculture experiences a crop with above average survival and growth, the price falls due to excess supply. During these times the cost of production often exceeds the income from sales (FAO, 2018). On the other hand, when shrimp aquaculture suffers losses due to disease or other factors, prices can spike for short periods. Unfortunately for producers suffering losses, the higher prices are typically not sufficient to maintain profitability (Samocha et al., 2004). The top three factors influencing shrimp profitability include disease loss, international market prices, and production costs (which are highly influenced by feed costs) (FAO, 2018).

Despite the variation due to market availability, the overall trend for shrimp prices has been on the rise. The wholesale market value of shrimp has ranged from $3,800-8,800 USD between 2000 and 2016 (SOFIA, 2018). Some government agencies have attempted to buffer the price fluctuations by setting a minimum price per kilogram of shrimp. The initial action came from the government of Andhra in April of 2018 and was quickly followed by the Department of Fisheries in Thailand in June of 2018, and others(FAO, 2018).

Global trade of shrimp continues to grow. Current projections estimate an 18% increase in production by 2020 (Mayes, 2018). In 2018, shrimp accounted for 6.2% of the total global fish trade but represented 16.1% of the total monetary value of the fish market. Shrimp represent the second largest aquaculture export species in terms of market value, only trailing combined counts of salmons, trouts, smelts (SOFIA, 2018). Shrimp aquaculture tends to occur in less developed nations or areas with below average

household incomes, and most of their product is exported (Gaille, 2018). The top exporters can be found in China (14.1%), Norway (7.6%) and Viet Nam (5.1%) (SOFIA, 2018). Over the last 24 years, shrimp production has risen 25%, in the same timeframe global trading of shrimp has increased 60% (Gaille, 2018). Losses in pond aquaculture often depend heavily on environmental conditions. Extreme heat or long stretches of below normal temperatures lead to systemic stress and allow for pathogen growth causing disease (Andriantahina et al., 2013). Most recently, disease and poor weather conditions have negatively impacted the productivity of nations like Thailand and China, while India and Ecuador have had fewer issues leading to great success in expanding production and filling the gap (SOFIA, 2018).

Top global importers of shrimp globally are the United States (15.1%), Japan (10.2%), China (6.5%) and Spain (5.2%) (SOFIA, 2018). The United States has increased shrimp imports by 40% in the last 5 years (Mayes, 2018). However, these imports pose risks as many countries have used banned antibiotics for disease control or employed poor food handling practices. Less than 2% of all United States imports are inspected by regulatory agencies, and of those 2% up to 35% are rejected due to the presence of illegal antibiotics or unsanitary holding conditions (Gaille, 2018; Greenberg, 2012).

2. Wild Caught Shrimp

2.1 History of Wild Harvest of Shrimp

Shrimp fishing has long history. The early methods of trawling date back to the 1800s when commercial fishery boats began to adapt nets to capture shrimp, as the

availability of finfish became depleted (Gillett, 2008). In 1890 Johan Hjort introduced, in Denmark, the first trawl technology specifically designed for the capture of shrimp (Cryer, Hartill, & O'Shea, 2002). In 1906 similar equipment, termed the Otter Trawl, was developed by Italian inventor Solicito Salvador and became the dominant method of shrimp harvest in the United States. Shrimp harvest in US waters continued at an ever-increasing rate until the 1950's when catch rates exceeded natural reproduction, and the fleets were forced to expand into Mexican waters. (Cryer et al., 2002). Fishing for shrimp continued to spread to alternate countries as market demand increased. Most fisheries were in temperate waters until the addition of tropical fisheries initiated in Africa in the 1960's (Cryer et al., 2002). In 2017 it was reported that the ocean harvest of shrimp was 270.79 million metric tonnes (Gaille, 2018). Shrimp harvest by commercial fishing provides an income to households across the world and can provide an alternate species to the market not currently available through aquaculture methods (Leung, 2006). The top producing countries of wild caught shrimp include: China (40%), India (12%), Indonesia (8%), Canada (5%), and the United States (5%) (SOFIA, 2018).

2.2 Methods of Wild Harvest of Shrimp

There are two basic methods for shrimp fishing, and both consist of pulling a conical tube of very fine netting behind a boat. The first method is known as dredging, where a single line is attached to the net; a portion of the net is lined with spikes to aid in digging into the ocean floor (Cryer et al., 2002). The second, more popular method is

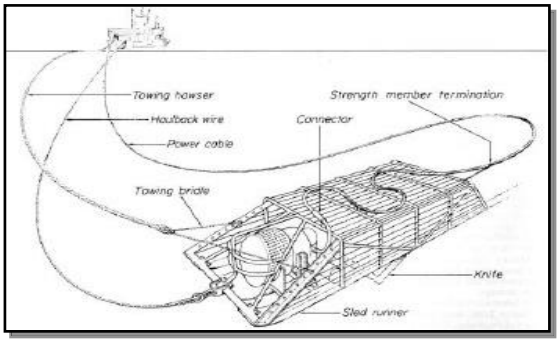

Figure 1 - Dredge

trawling. A trawling net is secured to the boat by two lines, with the mouth of the net held open with two or more boards, a portion of the net again drags across the ocean floor but does not possess spikes for digging (Dubay, Tokuoka, & Gereffi, 2010). Area covered by fishing can cover ten to over 100 meters in width of ocean floor in a single pass dependent on design, allowing for coverage of a minimum of 10km^2 of sea bed per trawler in a single fishing trip (Cryer et al., 2002).

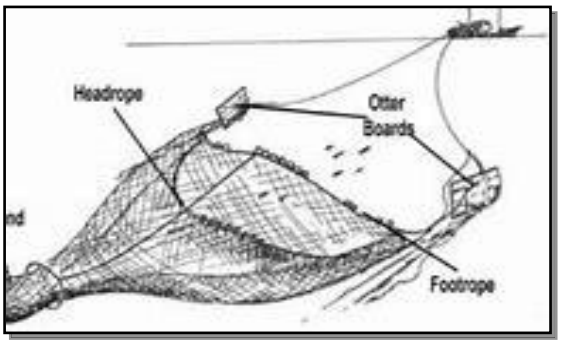

Figure 2 - Trawler

2.3 Adverse Effects of Wild Harvest of Shrimp

Unfortunately, commercial shrimp harvest methods have several disadvantages. Dragging the nets on the ocean floor is detrimental to the ecology of the ocean. It has been estimated that approximately 50% of the benthos on the ocean floor are destroyed with as few as seven passes with a trawler (Greenberg, 2012). There are very few restrictions on repeatedly fishing the same areas, and as a result several trawlers may pass over the same fishing grounds in any given day (Cryer et al., 2002).

Annual harvest amounts are another area of contention. The size and availability of shrimp are highly dependent on the season and growing conditions of the previous year (Greenberg, 2012). With the advanced fishing equipment of today, it can be relatively easy to over-harvest a region, and thereby negatively impact its future productivity. Consequently, the establishment of and adherence to harvest quotas is key to maintaining healthy and productive fisheries.

An alternate products of shrimp fishing are broodstock and post larvae shrimp that can be sold to operators of pond production systems. This production method employs an estimated one million people world-wide (Gillett, 2008). Over-harvest of broodstock and post larvae can also reduce the ability of a commercial fishery to produce sufficient quantities of market size shrimp for subsequent wild caught shrimp harvest (Greenberg, 2012).

2.4 Regulations on and Improvements to Wild Harvest Methods

Several laws and regulations have been implemented to help prevent other negative effects of shrimp fishing. The three most notable are the turtle conservation measures, anti-dumping tariffs related to bycatch, and eco-certification requirements for fisheries (Gillett, 2008; Greenberg, 2012). The Turtle Exclusion Device (TED) was invented by John Watson in the mid 1970's to prevent the capture of wild turtles. Successfully using these devices freed up to 97% of turtle caught in trawling nets (Lewison, Crowder, & Shaver, 2003). Even with the success in turtle conservation opposition existed as it is estimated 5-13% of the intended catch is lost during use (Lewison et al., 2003). Despite the loss in production turtle conservation regulations required the use of TED on shrimp nets in areas where turtles are known to inhabit the waters (Ellis, Kropp, Bridges, & Carpio, 2011). In 1989 regulations requiring the use of TED for imported and exporting products took effect causing a negative economic ripple in supply to the United States, as several countries were not compliant (Ellis et al., 2011). In 1998 the World Trade Organization determined the 1989 law violated fair trade laws

and banned its enforcement, thus allowing countries to freely trade shrimp caught without the use of TED (Lewison et al., 2003).

As noted earlier, the accidental capture of non-shrimp species during netting has been a long-standing concern. In some regions as much as 50-90% of the wild catch consists of non-shrimp species (known as bycatch) (Dubay et al., 2010; Ellis et al., 2011). Anti-dumping tariffs impose a fine for excessive bycatch. Several Bycatch Reduction Devices (BRD) have been introduced to reduce the volume of bycatch in the form of finfish (Gillett, 2008). The Magnuson-Stevens Fishery Conservation and Management Reauthorization Act of 2006 limits the shrimp-to-bycatch ratio to 4:1 up to 200 miles off shore in the United States and assesses a punitive fee of up to $100,000 USD or no more than 6 months in jail for those who exceed the limits (United States, 2006). The effectiveness of the Act is hard to determine as bycatch is poorly reported to date (Gillett, 2008).

Unfortunately, bycatch reduction devices are not always used, and even when they are, they do not eliminate bycatch. It is estimated that 1.8 million tonnes of marine life is wasted annually as a result of bycatch during commercial shrimp fishing, with some areas being larger contributors than others (Greenberg, 2012). Moreover, the industry often employs poor record keeping documenting bycatch species and counts. The lack of identification may lead to depletion of non-shrimp species and may affect the overall structure and health of the trophic webs (Gillett, 2008). To further reduce bycatch other methods have also been employed with some success, such as bans on trawling in specified zones and reduced fishing brought on by quotas (Gillett, 2008).

Eco-certification of fisheries is provided by non-government organizations to aid the fishing industry in developing and maintaining harvest methods to ensure survival and fishery sustainability. These third-party organizations aim to improve the fisheries for generations to come by imposing restrictions in order to achieve and maintain sustainable fisheries. The eco-certification provided by these organizations can be a useful marketing tool for companies targeting eco-conscious consumers, even though the restrictions may limit productivity (Ellis et al., 2011).

3. Aquaculture Production of Shrimp

3.1 History of Aquaculture Production

Due to increased market demand for shrimp and limited wild caught supplies, commercial production has shifted to aquaculture. The first reports of shrimp aquaculture occurred in the 1930's when incidental crops of shrimp were raised and harvested in India, Bangladesh, and Viet Nam after rice fields were intentionally flooded by coastal waters and stocked with wild shrimp fry (Alday-Sanz, 2010). Field workers subsequently harvested the adult shrimp as they attempted to escape to the sea after rice harvest. During the rearing process little was done to manage the ponds other than water exchange with the sea and addition of fertilizer to the system (Alday-Sanz, 2010).

The first breakthrough in shrimp cultivation occurred in 1934 when Dr. Motosaku Fujinaga of Japan developed shrimp spawning methods (Jobling, 2013). This discovery allowed the industry to produce and stock fry into ponds as needed, rather than relying on nature to provide sustainable stocks. Over the next three decades Fujinaga focused on perfecting methods for every step of production, from spawning to larval rearing and

finally grow out (Alday-Sanz, 2010). Due to his extensive contributions to the industry, Fujinaga is commonly referred to as the "Father of Shrimp Farming" (Alday-Sanz, 2010). In the 1960's the first commercial shrimp farm was established on the Seto Inland, Sea of Japan, and soon after the technology was transferred to the United States and Taiwan to continue the development of the industry (Alday-Sanz, 2010; Jobling, 2013).

In the United States this technology led to the establishment of two main research facilities: the Galveston Laboratory, later renamed the National Marine Fisheries Service, and the Marine Laboratory at the University of Miami (Jobling, 2013). The National Marine Fisheries Service focused on three species of shrimp *P. aztecus, P. duorarum* and *P. setiferus* (Jobling, 2013). Under the direction of principle investigators Harry Cook and Cornie Mock, research developed culturing methods in indoor tanks that used airlift systems to introduce dissolved oxygen. This production method is still used in commercial production (Alday-Sanz, 2010). The Marine Laboratory at the University of Miami focused on the species *P. duorarum*, using similar culturing methods as Fujinaga. The principal investigator, Won Tack Yang, studied under Fujinaga in Japan prior to moving to the United States (Alday-Sanz, 2010).

Several small commercial farms developed and failed in the 1960's due to poor growing conditions and sporadic disease. In 1968 Marifarms, in Panama City Florida, experienced frequent loss in production until they introduced a new species, *P. vannamei*, that was more resilient to disease and stress than previous species cultured (Jobling, 2013). Shortly after in 1970 the Ralston Purina Company, in Crystal River Florida, built and successfully operated the first semi-intensive farm and hatchery with the same species of *P. vannamei* (Alday-Sanz, 2010).

During the same timeframe in Taiwan the Tungkang Marine Laboratory was established to focus research on the species *P. monodon* (Jobling, 2013). Directing the laboratory was I. Chui Liao, another former student of Fujinaga. Liao was later referred to as the "Father of *P. monodon* Farming" for his advancements of shrimp culture utilizing the first intensive shrimp pond system (Alday-Sanz, 2010; Jobling, 2013).

The advancements in shrimp culture did not stop once commercial production was underway. In 1980, genetic selection of breeding pairs and family selection for production became primary foci of the United States Department of Agriculture – Marine Shrimp Farming Program (Jobling, 2013). The need for disease resistant shrimp had become a pressing issue as entire farms were succumbing to viral diseases at rates never seen before (A Bell & V Lightner, 1988).

By the 1990's, disease had become so prevalent that breeding efforts shifted to creating genetic lines of shrimp able to tolerate low salinity. This was done to allow shrimp aquaculture to move away from coastal areas where disease vectors were common (Jobling, 2013). These advancements in genetics led to a large production increase in China, where they have been able to use inland ponds to become the largest global shrimp producers since 2003 (Jobling, 2013). Soon after (2004), shrimp aquaculture in the United States started to decline and has remained in the same downward trend to the present (Jobling, 2013). A great deal of China's success in aquaculture is due to environmental responsibility, sustainable practices, product diversity, economy of scale, and business integration from producer to consumer (SOFIA, 2018).

Annual production of farmed shrimp has focused mainly on two species: *P. vannamei* and *P. monodon*. In 2017, 77% of all farmed fish were *P. vannamei*, which

equates to 42% of the combine wild shrimp catch and aquaculture production (Mayes, 2018). The main producer of farmed shrimp is Asia, utilizing man-made intensive ponds that provide two thirds of the global supply, with China accounting for 50% of all Asian production. The remaining third is produced in Latin America in similar production ponds ("Shrimp or prawn, wild or farmed: What's the difference? ," 2018).

Market locations for farmed shrimp vary depending on supply and price (Leung, 2006). Farmed shrimp are subject to price elasticity, meaning when supply exceeds demand the price of the shrimp will drop significantly (Leung, 2006). The opposite holds true as well, as significant reductions in supply generally result in price increases (Leung, 2006). Even with these price fluctuations, the base average price of shrimp has been rising for the past 48 years (SOFIA, 2018).

3.2 Methods of Aquaculture Production

By the 1970's three distinct methods of shrimp farming had emerged, extensive, semi-intensive, and intensive (Alday-Sanz, 2010). Each method is suited for different production environments and are employed across the globe (Alday-Sanz, 2010). A fourth method, called super-intensive, was developed in 2011 (Jobling, 2013).

3.2.1 Extensive Method of Shrimp Aquaculture

The extensive method has the lowest production capacity, and is typically found in ponds, costal impoundments, or natural estuaries (Jobling, 2013). In this method the production system is typically stocked with 2-5 shrimp per square meter of surface area with wild shrimp fry brought in by tidal water exchange (Alday-Sanz, 2010). The shrimp

survive on natural feeds, although in some cases Shrimp manures may be added to stimulate the development of additional natural feeds as needed. The extensive method only produces one crop per year resulting in an average maximum harvest of 400 kg of market shrimp per hectare per year (Alday-Sanz, 2010).

3.2.2 Semi-Intensive Method of Shrimp Aquaculture

The semi-intensive method utilizes drainable ponds that are 1-20 hectares in surface area and are 0.8-1.5 meters deep (Alday-Sanz, 2010). Post larvae are stocked at rates of 8-20 shrimp per square meter and are obtained from either wild caught and/or hatchery reared sources, depending on availability (Jobling, 2013). The shrimp are offered natural feeds found in the system, as well as supplemented with formulated feeds offered one or two times daily (Alday-Sanz, 2010). Semi-intensive systems require a daily water exchange with the ocean at a rate of 2-20%, dependent on the water quality (Jobling, 2013). Two cycles of production can occur annually, which can result in a maximum production of 4,000 kg of market shrimp per hectare per year (Alday-Sanz, 2010).

3.2.3 Intensive Method of Shrimp Aquaculture

The intensive production method uses drainable ponds, 0.1-1.0 hectare in surface area, with depths of 0.8-1.5 meters (Alday-Sanz, 2010). The ponds are stocked with 30-60 shrimp per square meter, with post larvae typically being supplied by a hatchery, although wild stock may also be utilized (Jobling, 2013). Formulated feeds are offered to the shrimp at least three times daily, with a maximum of five feedings per day (Jobling,

2013). These systems require mechanical aeration due to the high oxygen demand of the shrimp and the microbial biomass that colonize the systems (Jobling, 2013). Like the semi-intensive system, 2-20% water exchanges are required daily to maintain water chemistries and reduce stress to the shrimp (Alday-Sanz, 2010). These systems can produce two crops per year resulting in a maximum of 20,000 kg of market shrimp per hectare per year (Alday-Sanz, 2010).

A unique feature of the intensive production system is low salinity, which is typically between 2-6 parts per thousand (ppt), rather than the natural ocean salinity of 30-32 ppt (Prapaiwong & Boyd, 2012b). Otherwise, production parameters are very similar to that of the other shrimp aquaculture methods. The species of choice for production is again *P. vannamei*, due to its disease resistance and growth rates, and tolerance of a broad range of salinity (1-40 ppt) (Samocha et al., 2004). The intensive production method began in Taiwan in the 1990s, and by 1998 roughly 30% of their annual aquaculture production was derived from these systems (Prapaiwong & Boyd, 2012a, 2012b). Due to their success, countries such as Brazil, China, and the United States began accessing inland saline aquifers to establish low salinity ponds for intensive production (Prapaiwong & Boyd, 2012b). Currently in the United States there are active production research ponds in Alabama, Arizona, Florida, and Texas with the intent of producing a low-salinity commercial farming industry. (Prapaiwong & Boyd, 2012a).

3.2.4 Super-Intensive Method of Shrimp Aquaculture

The super-intensive method is the most productive aquaculture system. This method uses tanks of various sizes with average depths of 0.3-1.0 meters (Alday-Sanz,

2010). Tanks are constantly agitated at rates of 0.25 to 2.0 ft per second, and water is recirculated through some combination of treatment steps to remove chemical and physical contaminants (Alday-Sanz, 2010). Minimal wastewater discharge is required, with an average recycle of 90-99% of production water over a production cycle (Jobling, 2013). Tanks are stocked with hatchery post larvae at a rate of 65-90 shrimp per square meter of surface area (Jobling, 2013). Shrimp are fed more than five times daily with specially manufactured and formulated feeds to ensure complete nutrition (Jobling, 2013). These systems can produce 3-5 yearly crops, resulting in production of 45,000-75,000 kg market shrimp per hectare per year (Alday-Sanz, 2010).

3.3 Advantages of Aquaculture Production

There are several notable benefits to aquaculture production of shrimp. Farming has afforded the market a relatively consistent supply of shrimp available in predictable quantities and sizes (Ellis et al., 2011). Shrimp have shown the ability to grow two times faster in aquaculture systems compared to growth in the wild (Greenberg, 2012), and also exhibit fewer broken shells and other damage compared to wild caught shrimp (Dubay et al., 2010). In extensive systems shrimp require little more than a supply of natural feeds from ocean water exchange and the addition of animal manures if needed. Minimal inputs of extensive systems to achieve acceptable growth results in a low-cost production system (Alday-Sanz, 2010).

As shrimp density increases in progressively more intensive production systems, additional inputs/costs are necessary to maintain acceptable water quality and shrimp health (Dubay et al., 2010). However, these costs are offset by higher productivity. In

semi-intensive and intensive systems, shrimp can withstand some crowding while maintaining their health (Greenberg, 2012). In these systems naturally occurring populations of beneficial bacteria can be developed, or specific cultures of beneficial bacteria (probiotics) can be added to maintain acceptable water quality and reduce disease issues (Ellis et al., 2011).

In super-intensive systems, shrimp densities are sufficiently high so as to require use of water treatment systems in order to maintain acceptable water quality. In these recirculating aquaculture systems (RAS), mechanical filtration is used to remove solids. A biological filter is then used to remove nitrogenous wastes, followed by a sanitation step to remove excessive and/or pathogenic microbes that may lead to disease or stress (Jobling, 2013). Recirculating aquaculture systems help to control waste streams and are infinity expandable. Notable benefits of a RAS include high level of biosecurity and safe seafood supplies, energy conservation through heat retention, and water reclamation (Jobling, 2013).

Several multi-national financial institutions have invested in shrimp aquaculture as a method of reducing in under-developed countries (Jobling, 2013). Shrimp farming has provided a new income stream to low income households in developing economies, resulting in an overall decrease in poverty (Ellis et al., 2011). Farming is responsible for employing over a million people world-wide with the numbers growing each season (Gillett, 2008).

3.4 Disadvantages of Aquaculture Production

Shrimp aquaculture in ponds requires a relatively large footprint of land in relation to productivity. Moreover, land surrounding the ponds may be used to absorb the excess nutrient loads generated during shrimp production (Jobling, 2013). In some regions, large areas of mangrove forests were cleared in the 1980s for production (Leung, 2006). It is estimated ~10% of the world's mangrove forests were destroyed before this practice was stopped in the late 1990s (Greenberg, 2012).

Water quality can be adversely affected by aquaculture (Jobling, 2013). Shrimp production generates a significant amount of chemical and biological pollutants, and if this water is discharged into surrounding oceans it can potentially cause ecosystem damage (Ellis et al., 2011). The salt present in the wastewater typically precludes application on land, since salts inhibit plant growth. In cases where shrimp wastewater has been land applied, these lands are often converted to additional ponds due to salt contamination (Ellis et al., 2011).

Recirculating aquaculture systems are a partial solution to this problem, by minimizing the overall volume of wastewater discharge. However, recirculating systems can be costly to establish and operate (Jobling, 2013). Commercial RAS have been attempted several times over the last two decades only to fail (Jobling, 2013). Efforts to simplify and reduce costs of RAS systems are underway to water quality outcomes that will be economical (Jobling, 2013).

Shrimp loss due to disease is also a potential disadvantage of these systems, especially when they are operated at higher densities which results in higher levels of stress on the shrimp (Greenberg, 2012). The increased stress may result in greater

susceptibility to bacterial or viral diseases, leading to high mortality (Ellis et al., 2011). Shrimp aquaculture can also affect or be affected by disease in natural ecosystems. In extreme cases, diseased shrimp have escaped production ponds and exposed shrimp in native waters to disease (Samocha et al., 2004). In 1997 off the coast of Texas, wild white and brown shrimp tested positive for the viral disease WSSV common to the aquaculture disease experienced in the area in the same time frame (Galitzine, Morgan, & Harvey, 2009). Sporadic episodes of bacterial and viral diseases in natural shrimp populations have also reduced the expansion of commercial shrimp farming (Samocha et al., 2004). As the industry began to develop in the 1980s several new diseases had been discovered due to infection from wild populations (Lightner et al., 2012). The spread of MBV can be traced from Asia, Australia, and Africa to the Americas by the transport of wild caught infected shrimps for aquaculture use (Lightner et al., 2012).

To combat disease risk, antibiotics have been routinely added to some shrimp aquaculture systems. An unfortunate consequence of this practice can be development of resistant strains of bacterial pathogens that can then be transferred to other native or aquaculture populations of shrimp, or even to humans upon ingestion of contaminated shrimp (Leung, 2006). Currently only the United States has regulations on the use of antibiotics in aquaculture (Ellis et al., 2011).

4. Microbiome

4.1 Introduction

The term microbiome is defined as the collection of microbial species specific to any given environment (E. Li et al., 2018). In the past decade, emphasis has been placed

on developing an understanding of the relationships between the host and the gut microbiome in humans and terrestrial animals (E. Li et al., 2018). In 2008 the Human Microbiome Project began, with the goal of determining the bacterial composition of the human gut, and understanding the relationships between host, microbiome and potential pathogens (Lloyd-Price et al., 2017). Samples collected from several individuals were analyzed using metagenomics. Researchers discovered that the gut microbiome varied depending on diet and geographical distribution (Lloyd-Price et al., 2017). Researchers also observed that the same main metabolic functions were performed by the microbiome, even though different families of bacteria were present (Lloyd-Price et al., 2017). The team concluded that the gut microbiome evolves for the benefit of the host (Lloyd-Price et al., 2017). A second round of studies in the Human Microbiome Project was completed in 2017, and researchers noted that the gut microbiome evolved at a much faster rate for people exposed to a more variable environment (Lloyd-Price et al., 2017).

The gastrointestinal tracts of terrestrial and aquatic species also house symbiotic communities of microorganisms. Their gut microbiome also plays a vital role in developing the host's physiology and immune response, while maintaining nutritional health, regulating metabolic processes, and synthesizing vitamins for the host (Cornejo-Granados et al., 2017). The abundance and diversity of the microbiota is affected by feed intake, probiotics, prebiotics, hormone secretion, stress, antibiotic use, developmental stage, environmental conditions, physiological conditions, host metabolism, and immune response (Cornejo-Granados et al., 2017). Changes in the gut microbiome dependent on these factors have been researched in several shrimp species including: *Penaeus*

monodon, Fenneropenaeus chinensis, Penaeus penicillatus, Penaeus merguiensis and *Litopenaeus vannamei* (Cornejo-Granados et al., 2017).

In the past decade shrimp production has been impeded by disease, poor growth due to diet changes and environmental conditions, and antibiotic overuse that altered the abundance and composition of the natural microbiota (E. Li et al., 2018). Little is known about how aquaculture methods affect the shrimp microbiota (Cornejo-Granados et al., 2017). Some benefits have been shown in the use of probiotics and prebiotics to reduce stress, aid in the efficiency of plant protein digestion, and counter the adverse effects of antibiotics on the microbiome. However, there are abundant opportunities to further explore how to influence the gut microbiome to enhance shrimp aquaculture (E. Li et al., 2018).

4.2 Methods to Study the Microbiome

Early research into the shrimp involved culturing samples of gut contents in enriched media, and then identifying the microbial isolates (Huang et al., 2016). Using this method, 111 bacterial species have been identified from 13 taxonomical groups, ultimately revealing seven dominant groups of bacteria present in the shrimp gut: *Photobacterium, Vibrio, Aeromonas, Xanthomonas, Agrobacterium, Bacillus* and the family Enterobacteriaceae (E. Li et al., 2018). Other molecular approaches soon followed, including denaturing gradient gel electrophoresis (DGGE) and clone libraries (Cornejo-Granados et al., 2017). These methods worked well for known species of interest, but 98-99% of bacteria in the gut was and continues to be unculturable, and therefore remained undefined (E. Li et al., 2018).

The development of 454 pyrosequencing in the human microbiome project proved to be a powerful and efficient method to determine abundance and composition of any microbiome (Huang et al., 2016). Following extraction of 16S rRNA from bacterial samples, DNA copies are generated and amplified by polymerase chain reaction (PCR), and then sequenced to identify the source of the DNA (Huang et al., 2016). As microbiome analysis has evolved, the 16S rRNA gene sequences discovered have been loaded into public databases and are available to conduct comparative analyses. Using pyrosequencing, scientists were able to determine *Proteobacteria* were the most prevalent microbial group in shrimp, not unlike other aquatic species (E. Li et al., 2018). However, the investigation into the shrimp microbiome is still in its' infancy and various studies are still needed to complete the microbiome profile (E. Li et al., 2018).

4.3 The Shrimp Microbiome

It has been discovered that the microbiome of shrimp and other aquatic species varies greatly from that of terrestrial animals – with aquatic species being dominated by *Proteobacteria,* rather than *Firmicutes* and *Bacteroides* that are common in terrestrial animals (Xiong et al., 2017). Secondary contributors to the shrimp microbiome are *Firmicutes, Bacteroides,* and *Actinobacteria,* but the relative abundance of each is highly dependent on the environment and diet (E. Li et al., 2018).

Shifts in the microbiome are caused by changes in rearing conditions, trophic levels, developmental stages, and health status, which is referred to as the ecological theory (Xiong et al., 2017). These shifts can occur via four processes: selection, drift, diversification, and dispersal of speciation or mutation (Xiong et al., 2017). Selection is

the process by which the environment of the shrimp's gut is colonized by species found in the living environment (E. Li et al., 2018). Selection can be seen by the comparison of the microbiota of the wild shrimp gut compared to a pond raised shrimp. Pond raised shrimp microbiomes are less diverse due to the limited diversity of the pond sediment and water compared to wild environments (Cornejo-Granados et al., 2017). Drift occurs when the colonization of the gut moves or drifts to adapts due to an external input (Xiong et al., 2017). Drift can be observed in the shrimp gut when the diversity and abundance of microbial species shifts from the live feeds to commercial diets used in aquaculture (E. Li et al., 2018). Diversification occurs when the microbiota in the gut progresses from a large abundance of a few strains of bacteria to a greater assortment of bacteria with lower abundances that can perform similar functions (E. Li et al., 2018). As the juvenile shrimp develops a health gut diversifies allowing for a better defense against disease development (De Schryver & Vadstein, 2014). In the event the pathogenic species can out compete one or more of the species in the gut the diversification limits susceptibility (De Schryver & Vadstein, 2014). Lastly, dispersal of speciation or mutation is a process by which bacteria will move to sections of the environment in which it can fill a niche or will mutate in order to find an ecological advantage over the other strains present (Xiong et al., 2017). Dispersal in an aquatic species gut is typically limited as the environment, feed, and shrimp gut maintain constant contact (De Schryver & Vadstein, 2014). The microbiome evolves so as to develop a symbiont relationship with the host, meaning that both entities gain from the relationship (Xiong et al., 2017).

Three main probiotic bacterial genera have been identified in the gut microbiome of healthy shrimp: *Lactobacillus* at 1.0% of total abundance, *Streptococcus* at 0.93% of

total abundance, and *Bacillus* at 0.37% of abundance (E. Li et al., 2018). Although these bacteria do not represent the typical abundance in the gut, only representing 2.5% of the total, they have been shown to promote health and growth in differing species. As an example, *Bacillus spp.* in *Penaeus japonicas* improved immunity, whereas the same species in *Litopenaeus vannamei* promoted growth through improved nutrient digestibility (Huang et al., 2016).

Opportunistic pathogens are always present in the microbiome at low levels, and they are benign under normal culture conditions (Huang et al., 2016). Disease only occurs when conditions become suitable for the pathogens to take control of the gut environment, and this is usually due to external stress or poor diet (E. Li et al., 2018). Pathogens that commonly colonize shrimp are members of the family Enterobacteriaceae, including: *Pseudomonas, Flavobacterium, Echerichia, Aeromonas, Vibrio, Rickettsia, Shewanella,* and *Desulfovibrio* (E. Li et al., 2018).

4.4 Effects of Age and Physiological Development on the Gut Microbiome

The initial development of the gut microbiome for shrimp is acquired mainly from artemia, a live feed offered just days after hatching (E. Li et al., 2018). Bacteria phylum that dominate the gut at this stage include *Proteobacteria, Bacteroidetes* and *Actinobacteria* (E. Li et al., 2018). The microbiome then continues to shift with developmental stages up to approximately ninety days of age when commercial feeds are offered, and the diet is regulated, which is also the point at which the shrimp become increasingly susceptible to bacteria disease (E. Li et al., 2018). Even with the diet changes there is strong evidence that the microbiome present at any given age is highly

dependent on the developmental physiology of the host and variation form the normal microbiome may indicate progression of diseased states (Xiong et al., 2017).

4.5 Effects of Nutrition on the Gut Microbiome

Functionality of a shrimp's digestive system is strongly dependent on the establishment of a gut microbiome that is able to break down macro and micro nutrients (Huang et al., 2016). Lack of development of an acceptable gut microbiome can lead to malnutrition and failure to thrive. Once shrimp have become weakened by poor nutrition, they are highly susceptible to disease (Xiong et al., 2017).

The key to understanding shrimp nutrition is to under the composition and roles of the microbiota in relation to the feed materials supplied (Cornejo-Granados et al., 2017). Manufactured diets have created a notable shift in the shrimp gut microbiome, largely dependent on the types of lipids and proteins, as well as copper content (E. Li et al., 2018). Nutritionist have shifted from using animal proteins, which are costly and in limited supply, to plant sourced proteins that are lower cost and more widely available (Huang et al., 2016; E. Li et al., 2018). A major difference is that plant protein sources also frequently contain higher levels of carbohydrates than animal proteins. Shrimp inherently have difficulty digesting carbohydrates, as their microbiome contains only a small percentage of carbohydrate metabolizing *Bacteroidetes* (E. Li et al., 2018). The microbiota in cultured shrimp fed such diets has quickly shifted to accommodate the higher carbohydrate levels (Huang et al., 2016).

4.6. Effects of Environment and Water Quality on the Gut Microbiome

Aquatic systems are capable of inoculating the shrimp gut by the inclusion of bacteria found in sediment and water (Cornejo-Granados et al., 2017). There are over ninety bacterial genera commonly reported in pond sediment, culture water, and the shrimp gut, with the highest similarities between the gut and sediment profiles (E. Li et al., 2018). The most abundant genera in sediment and the shrimp gut include *Lactobacillus, Streptococcus,* and *Bacillus* (E. Li et al., 2018). This finding has motivated operators of many shrimp production systems to add these genera as probiotics.

The shrimp gut microbiome is responsive to environmental conditions. For example, when the temperature of a system rises to levels that stress the shrimp, the gut microbiota shifts rapidly to a higher percentage of pathogens (E. Li et al., 2018). Low salinity can also cause shrimp stress, resulting in an increase in pathogenic species in the gut (E. Li et al., 2018). Recently *Actinobacteria* has been discovered as a key bacterial phylum in the gut of *Litopenaeus vannamei,* which allows it to thrive in low salinity by preventing the overgrowth of pathogens (E. Li et al., 2018).

4.7 Shrimp Health and Disease as Influenced by the Gut Microbiome

Most shrimp health problems are nutritionally related and have been exacerbated by the shift in commercial diets from fish meals and oils to plant based products that contain higher levels of carbohydrates and fiber, which are not native to shrimp diets (E. Li et al., 2018). This dietary change has shifted the microbiome to contain higher levels of *Firmicutes,* which can utilize fiber as a carbon source to produce short chain fatty

acids that promote intestinal health. However, *Firmicutes* are selective to the source of fiber, and some fiber sources are poorly metabolized (E. Li et al., 2018).

Abundance of pathogenic bacteria in the shrimp gut will change over the life of the shrimp as the diet composition changes, thus it is important for commercial diets to be formulated to balance physiological and nutritional needs to maintain health (E. Li et al., 2018). Recent studies have shown that stress will reduce growth of the natural microbiota and allow pathogenic bacteria to thrive, leading to an overgrowth and disease (Xiong et al., 2017). For example, a depletion of *Lactobacillus*, and increase in pathogens can result in a loss of shrimp weight (E. Li et al., 2018). A possible solution to this shift would be the addition of probiotics or beneficial bacteria to protect against the development of pathogens and enhance the health (Huang et al., 2016).

CHAPTER ONE

Impact of Aquaculture Practices on Intestinal Bacterial Profiles of Pacific Whiteleg Shrimp *Litopenaeus vannamei*

1. Abstract

Considering the crucial role of the gut microbiome in animal health and nutrition, solutions to shrimp aquaculture challenges, such as improving disease resistance and optimizing growth on lower cost feeds, may lie in manipulation of their microbial symbionts. However, achieving this goal will require a deeper understanding of shrimp microbial communities and how their composition is influenced by diet formulation, environmental conditions, and host factors. In this context, the current study investigated the intestinal bacterial communities of the Pacific whiteleg shrimp (*Litopenaeus vannamei*—the most widely aquaculture-farmed shrimp worldwide) reared in indoor aquaculture facilities and outdoor pond systems. While samples showed very consistent intestinal bacterial community profiles within each production system, major differences were uncovered between the two practices. Indeed, bacteria affiliated with Rhodobacteraceae (Proteobacteria) and Actinobacteria were significantly more abundant in indoor samples (84.4% vs. 5.1%; 3.0% vs. 0.06%, respectively), while Vibrionaceae (Proteobacteria), Firmicutes, Fusobacteria and Cyanobacteria were predominant in pond samples (0.03% vs. 44.8%; 0.7% vs. 36.0%; 0.0% vs. 7.9%; 0.001% vs. 1.6%, respectively). Accordingly, the abundance of 11 of the 12 most prominent Operational Taxonomic Units (OTUs) were found to be statistically different between the two production environments. Together, these results indicate that aquaculture practices greatly influence the intestinal bacterial profile of the whiteleg shrimp, and further

suggest that bacterial communities of this economically important crustacean could be effectively manipulated using diet composition or environmental conditions.

2. Introduction

Shrimp is one of the most important seafood traded worldwide, with more than 3.4 million tons marketed each year at an estimated wholesale price ranging between $3800 and $8800 USD per ton (SOFIA, 2018). As the global human population continues to grow, shrimp supplies will need to double in the next 20 years in order to meet future demand (Jobling, 2013). As wild harvest capture has grown stagnant (Gillett, 2008), aquaculture has become the most viable alternative to meet current and future shrimp market demands (Jobling, 2013). Indeed, 55% of the annual global shrimp supply in 2018 was produced by farming (Gaille, 2018), suggesting that aquaculture has the capacity to provide consumers with a consistent and reliable supply of product (Ellis et al., 2011). While still in its infancy, shrimp farming has shown great potential for high productivity at reduced costs. For instance, aquaculture-raised shrimp have shown twice the growth rates of wild stocks, indicating great potential to further increase production (Greenberg, 2012). Due to its tolerance to a wide range of salinities and temperatures, whiteleg shrimp (Litopenaeus vannamei), also known as Pacific white shrimp or king prawn, is the most widely farmed shrimp worldwide (Cheng, Hu, Liu, Zheng, & Qi, 2006; E.-C. Li et al., 2017).

Outdoor ponds with close access to ocean water represent the most popular and basic design for shrimp farming. Regular exchanges with ocean water are used to both replenish food sources for growing shrimp and to evacuate waste from the ponds.

Variations amongst production systems are typically a function of stocking density, which primarily affects ocean water inputs needed to maintain water quality (Dubay et al., 2010). Since they require minimal inputs to ensure growth, ponds represent an attractive, low-cost production system (Alday-Sanz, 2010). However, the impact of pond-based production on the environment, as well as risks for pathogen outbreaks, are cause for concern. Effluent from shrimp production ponds is a significant source of chemical and biological pollutants in ocean waters that can harm natural aquatic habitats that are sensitive to excessive nutrient loads (Ellis et al., 2011; Jobling, 2013). The exposed nature of open ponds means they typically have limited protection against exposure to pathogens, with high density ponds at greater risk of experiencing outbreaks as a result of increased pollution and stress conditions (Greenberg, 2012). Heavy losses in shrimp production due to disease have historically been a defining feature of this industry (Samocha et al., 2004), particularly from pathogens of the Enterobacteriaceae family, which include species affiliated to Pseudomonas, Flavobacterium, Escherichia, Aeromonas, Vibrio, Rickettsia, Shewanella and Desulfovibrio (E. Li et al., 2018). Once infected, ponds themselves present a risk for contaminating wild populations residing in nearby native waters (Samocha et al., 2004). Treatment of pond water with antibiotics is a common strategy to mitigate risk of disease, but this practice can lead to the selection of resistant microbial strains that could be transferred to the human food supply (Leung, 2006), as well as surrounding natural waters. The trend for stricter antibiotic regulations worldwide in livestock production also indicates that alternative methods in aquaculture will be needed in the near future.

In contrast to ponds, indoor facilities for shrimp farming allow tighter biosecurity control and help lower the environmental footprint of aquaculture. While they require more costly investments in infrastructure, such as recirculating water systems, indoor facilities can dramatically minimize exposure to environmental pathogens, provide better control of water quality with reduced impact on the environment, and provide safer seafood products that are free from food-borne contaminants (Jobling, 2013). Besides facility costs, the other main disadvantage of indoor aquaculture is the need to formulate diets that optimize shrimp growth at reduced costs. Ingredients have traditionally been derived from fish byproducts such as fish meal and fish oils, but the higher cost of these feedstuffs and other animal protein sources has motivated the use of plant-derived proteins (Huang et al., 2016; E. Li et al., 2018). However, because plant products are not natural components of shrimp diets and contain high levels of carbohydrates and anti-nutritional factors that shrimp have not evolved to digest, high inclusion levels may result in sub-optimal growth and poor health (E. Li et al., 2018).

Considering the overarching importance of the intestinal microbiome in animal health and nutrition (Cornejo-Granados et al., 2017), manipulation of beneficial microbial communities in the shrimp gut may provide solutions to improve resistance to pathogens without prophylumctic use of antibiotics, as well as to optimize growth on alternative protein sources. Research to date has found that the gut bacterial profile in healthy shrimp consists primarily of Proteobacteria, consistent with marine fish (Califano et al., 2017), which is in stark contrast to the microbiome of terrestrial animals in which Firmicutes and Bacteroides are typically dominant (Xiong et al., 2017). Indeed, these latter phylum have so far been found to be minor components of the shrimp gut microbiome, and their

abundance appears to be highly dependent on local environmental conditions and diet composition (E. Li et al., 2018). There is limited knowledge to date on the effect of aquaculture practices on the shrimp gut microbiome (Cornejo-Granados et al., 2017). For instance, biosafety measures to prevent pathogen outbreaks may also inadvertently reduce or prevent colonization of indoor raised shrimp with beneficial bacteria found in natural environments (Cornejo-Granados et al., 2017). Since early gut microbial colonization events can impact the future performance or productivity of an animal, proper microbiome development may be detrimentally altered in aquaculture raised shrimp (Huang et al., 2016). Conversely, gut bacterial communities of shrimp have been shown to adapt to commercially formulated diets, indicating that the shrimp microbiome can be manipulated through dietary ingredients (Huang et al., 2016).

Since diet and host genetics, as well as a number of environmental conditions such as water temperature, salinity, and sulfide concentrations, can affect the gut microbiome composition of whiteleg shrimp (E. Li et al., 2018), we hypothesized that the intestinal bacterial communities of this highly farmed aquatic species would differ between the two main types of aquaculture systems. To this end, the study presented in this report compared the intestinal bacterial profile of whiteleg shrimps raised in an indoor facility with individuals of the same species collected from two pond systems. Within each production system, which were operated under their respective normal practices, samples showed very consistent taxonomic profiles and gut bacterial community structures. However, major differences in taxonomic affiliations and Operational Taxonomic Unit (OTU) profiles were uncovered between shrimp raised in an indoor facility and shrimp raised in ponds. Together, these results indicate that

aquaculture farming conditions greatly influence the composition of the white-leg shrimp gut microbiome.

3. Materials and Methods

3.1. Sample Collection and Harvesting of Shrimp Intestinal Tissue

Shrimp were captured from three different production environments (as described below). Intestinal tissue was harvested from each animal using the following procedure. The telson was removed with scissors distal to the sixth abdominal segment, then the posterior end of the carapace was lifted to expose the hepatopancreas and the proximal end of the gut. The intestine was then excised with sterile tweezers starting at the hepatopancreas on through to the hind gut. Each sample consisted of intestinal tissue pooled from five individual shrimp from the same population (see description below) to ensure sufficient material was available for DNA extraction. All harvested intestinal tissue samples were stored at −20 °C until DNA extraction.

3.2. Study Site for the Indoor-Raised Shrimp

The trū Shrimp Innovation Center (330 3rd Street, Balaton, MN, USA; 44.2° N 95.8° W) is a research campus designed to industrialize indoor aquaculture techniques for shrimp production. The facility contains nearly 200 clearwater and biofloc research tanks, as well as other commercial production tanks of various sizes and configurations. Indoor-raised shrimp were maintained at 28 ± 1 °C in temperature-controlled tanks. Water management was carried out using separate indoor recirculating aquaculture systems, one for each tank, utilizing fresh water processed by reverse osmosis then mixed at 28 g

Marinemix (Marine Enterprises International, LLC., Baltimore, MD, USA) to one-liter production water. Total ammonia nitrogen (TAN) levels were maintained at less than 3.0 mg/mL, nitrite levels below 4.5 mg/mL, and nitrate levels never exceeded 100 mg/mL. Evaporated water was replaced with fresh water as needed to maintain salinity at 28 ppt. Stocking densities were maintained in the standard range of intensive production systems at 30–60 shrimp per cubic meter, with feed offered continuously. All culture tanks were supplemented with a commercial blend of probiotic bacteria (BioWish 3P, BioWish Technologies, Cincinnati, OH, USA) containing Pediococcus acidilactici, P. pentosaceus, Lactobacillus plantarum and Bacillus subtilis. The probiotic was provided as a daily dose of 0.025 g/100 L, which was manually administered over the course of a 24-h period.

Fourteen healthy indoor-raised aquaculture samples were obtained from six production tanks fed three separate diets at three different time points (see Table 1 for sample description). The diets fed all contained 35% crude protein, and consisted of Production 35% (Rangen Inc., Buhl, ID, USA), Hyper-Intensive 35 (Zeigler Bros. Inc. Gardners, PA, USA) and tSC (tSC 35%, tSC Grow Out, Balaton, MN, USA). Data from partial nutrient composition of diets used is presented in Table 2. As they did not require long distance transportation, no preservative was added to intestinal tissue samples from indoor-raised shrimp, and they were immediately stored at −20 °C after dissection.

Table 1. Culture tank assignment, diet, time points, average length, and weight of shrimp sampled from the indoor production facility.

Tank	Diet	Time Point (day)	Sample	Average Weight (g)	Average Length (cm)
ST1	Rangen	34	I-R-34[a]	1.304	5.2
ST2	Zeigler	34	I-Z-34[a]	1.453	5.3
ST3	tSC	34	I-t-34[a]	1.248	5.0
STA	Rangen	34	I-R-34[b]	1.872	5.8
STB	Zeigler	34	I-Z-34[b]	1.278	5.1
STC	tSC	34	I-t-34[b]	1.258	5.1
ST1	Rangen	44	I-R-44[a]	2.357	6.3
ST2	Zeigler	44	I-Z-44[a]	2.381	6.3
ST3	tSC	44	I-t-44[a]	2.693	6.6
STA	Rangen	44	I-R-44[b]	2.387	6.3
STB	Zeigler	44	I-Z-44[b]	2.428	6.4
STC	tSC	44	I-t-44[b]	2.039	6.0
ST3	tSC	70	I-t-70[a]	7.256	8.8
STC	tSC	70	I-t-70[b]	8.868	9.2

The [a] or [b] are indicative of replicates of the same treatment

Table 2. Partial nutrient content of aquaculture diets[a]. Values are expressed as percentage (%).

Diet Name	Protein[b]	Fat	Fiber	Ash	Manufacturer
Hyper-Intensive 35	35 (M,P)	7	2	13	Zeigler
Production 35%	35 (M,P)	8	3	15	Rangen
tSC 35	35 (P)	9	2	12	tSC
Bumper Crop	35 (M)	8	3	12	Vimifos

[a] Formulation of all diets presented in this table are proprietary. [b] Primary source of protein ingredients in the diet: marine animal (M), plant (P).

3.3. Pond-Raised Shrimp

Samples from healthy, pond-raised shrimp were obtained from two farms. Ten samples were collected from a shrimp farm located on the coast of Kino Bay (Sonora, Mexico—samples labeled 'KB-'), and five other samples were obtained from a farm located near Obregon (Sonora, Mexico—samples labeled 'Ob-'). Ponds averaged a depth of 1.5 meters, with stocking densities maintained within a range typical of intensive production systems (30–60 shrimp/m^3). Water chemistry testing was conducted weekly to monitor levels of TAN, nitrite, nitrate and alkalinity. These parameters were used to determine the rates of water exchange to maintain water quality, which ranged from 0% to 20%. Both sites fed the same commercial diet (Bumper Crop, Vimifos S.A. de C.V., Guadalajara, Jalisco, Mexico; data from partial nutrient composition is presented in Table 2), with the Obregon farm ponds receiving a daily dose of phytochemicals as a dietary supplement. Feed was offered at scheduled times during the day (5 a.m., 12 p.m. and 5 p.m.). Pond sampled shrimp were harvested at approximately day 80 of age, then dissected on site. Intestinal samples were preserved in 100% ethanol for transportation, and then stored at −20 °C upon arrival at the host laboratory.

3.4. Wild-Caught Shrimp

It is very challenging to obtain wild-caught shrimp that are not degraded from storage or preservation methods on a fishing vessel. Therefore, only one wild-caught sample (five shrimp from the same catch) of high quality could be obtained for this study. These shrimp of undetermined age and natural diets were caught in the Gulf of California (34 to 36 ppt salinity). Dissected intestinal samples were preserved in 100% ethanol for

transportation, then stored at −20 °C upon arrival at the host laboratory. Because a single sample increases the risk of potential bias, the microbiome composition of the wild caught sample was only used as a qualitative reference.

3.5. Microbial DNA Isolation and PCR Amplification

Microbial DNA was isolated from shrimp intestinal samples using the repeated bead beating plus column method, as described by Yu and Morrison (Yu & Morrison, 2004). The V1–V3 region of the bacterial 16S rRNA gene was PCR-amplified using the 27F forward (Edwards, Rogall, Blocker, Emde, & Bottger, 1989) and 519R reverse (Lane et al., 1985) primer pair. PCR reactions were performed with the Phusion Taq DNA polymerase (Thermo Scientific, Waltham, MA, USA) under the following conditions: hot start (4 min, 98 °C), followed by 35 cycles of denaturation (10 s, 98 °C), annealing (30 s, 50 °C) and extension (30 s, 72 °C), then ending with a final extension period (10 min, 72 °C). PCR products were separated by agarose gel electrophoresis, and amplicons of the expected size (~500 bp) were excised for gel purification using the QiaexII Gel extraction kit (Qiagen, Hilden, Germany). For each sample, approximately 400 ng of amplified DNA were submitted to Molecular Research DNA (MRDNA, Shallowater, TX, USA) for sequencing with the Illumina MiSeq 2X300 platform to generate overlapping paired end reads.

3.6. Computational Analysis of PCR Generated 16S rRNA Amplicon Sequences

Unless specified, sequence data analysis was performed using custom written Perl scripts (available upon request). Raw bacterial 16S rRNA gene V1–V3 amplicon

sequences were provided by Molecular Research DNA as assembled contigs from overlapping MiSeq(2X300) paired-end reads from the same flow cell clusters. Reads were then selected to meet the following criteria: presence of both intact 27F (forward) and 519R (reverse) primer nucleotide sequences, length between 400 and 580 nt, and a minimal quality threshold of no more than 1% of nucleotides with a Phred quality score lower than 15.

Following quality screens, sequence reads were aligned, then clustered into Operational Taxonomic Units (OTUs) at a genetic distance cutoff of 5% sequence dissimilarity (Opdahl, Gonda, & St-Pierre, 2018). While 3% is the most commonly used clustering cutoff for 16S rRNA, it was originally recommended for full length sequences, and may not be suitable for the analysis of specific subregions since nucleotide sequence variability is not constant across the entire length of the 16S rRNA gene. In this context, if 3% is a commonly accepted clustering cutoff for V4 or V4–V5 regions, which are the least variable of the hypervariable regions, then a higher cutoff should be used for the V1–V3 region, since V1 is the most variable region of the 16S rRNA gene. OTUs were screened for DNA sequence artifacts using the following methods. Chimeric sequences were first identified with the chimera.uchime and chimera.slayer commands from the MOTHUR open source software package (Schloss et al., 2009). Secondly, the integrity of the 5′ and 3′ ends of OTUs was evaluated using a database alignment search-based approach; when compared to their closest match of equal or longer sequence length from the NCBI nt database, as determined by BLAST (Altschul et al., 1997), OTUs with more than five nucleotides missing from the 5′ or 3′ end of their respective alignments were discarded as artifacts. Finally, single read OTUs were removed from the analysis.

After the removal of sequence chimeras, artifacts, and singleton OTUs, taxonomic assignment of OTUs was determined using a combination of RDP Classifier (Wang, Garrity, Tiedje, & Cole, 2007) and BLAST (Altschul et al., 1997). The List of Prokaryotic Names with Standing in Nomenclature (LPSN http://www.bacterio.net) was also consulted for information on valid species belonging to taxa of interest (Parte, 2014).

3.7. Computational Analysis for Alpha and Beta Diversity

Using custom Perl scripts, each dataset was randomly rarefied to 5000 reads, with the 50 iterations created for each sample used to create 'shared'-type formatted files. All subsequent steps were performed using commands in MOTHUR (Schloss et al., 2009). Chao1, Shannon and Simpson indices, as well as observed OTUs and coverage, were determined from the shared files using summary.single. For Principal Coordinate Analysis (PCoA), Bray-Curtis distances were first determined using summary.shared, which were then used as input for the command pcoa. Principal Components 1 (PC1) and 2 (PC2), representing the highest level of variation, were plotted using Microsoft® Excel.

3.8. Statistical Analysis

An independent t-test was used to compare the relative abundance of bacterial taxonomic groups and OTUs between different production systems, respectively (GraphPad Software, https://www.graphpad.com/quickcalcs/ttest1.cfm). The means of two groups were considered to be significantly different when $P \leq 0.05$.

3.9. Accession Numbers for Next Generation Sequencing Data

Raw sequence data are available from the NCBI Sequence Read Archive under Bioproject PRJNA522274 and SRA accession SRP185856. Accession numbers for individual samples are provided in Supplementary Table S1.

4. Results

4.1. Comparative Analysis by Taxonomic Composition

A total of 699,259 high quality and chimera/artifact-free reads were used for analysis, with an average of 22,698 ± 1744 reads per sample for the indoor-raised shrimp samples, 24,796 ± 4223 reads per sample for the pond samples, and 9539 reads for the wild-caught sample. A total of 6988 sequence reads, ranging between 22 and 1025 reads per sample, were designated as 'unclassified' because they could not be assigned to a known phylum.

Gut bacterial communities of indoor- and pond-raised whiteleg shrimp were found to be very different ($P < 0.05$) in taxonomic profiles. Proteobacteria was overall the most dominant phylum across the samples analyzed, but significantly higher levels were found in indoor samples (88.6%) compared to ponds (51.8%) (Table 3). More discernable differences were observed between the two aquaculture environments at the family level, as bacterial communities from indoor samples were primarily composed of members of the Rhodobacteraceae family (84.4%), while Vibrionaceae were found to be the most abundant in pond samples (44.8%). In addition, Firmicutes, Fusobacteria and Cyanobacteria were found in much higher abundance in pond samples (36.0%, 7.9% and 1.6%, respectively) compared to indoor samples (0.7%, 0.0% and 0.001%, respectively),

in contrast to Actinobacteria, which were more highly represented in indoor samples (3.0% vs. 0.06%). While the limited number of wild-caught samples did not allow a statistically based comparison, a qualitative assessment of abundance values revealed that the overall taxonomic composition of wild-caught shrimp was more similar to pond-raised shrimp than shrimp farmed in indoor facilities (Table 1, Figure 1).

Figure 1. Venn diagram showing the number of shared and unique OTUs from the intestine of indoor-raised, pond-reared and wild caught white leg shrimp. Also shown is the proportion of sequence reads for each category.

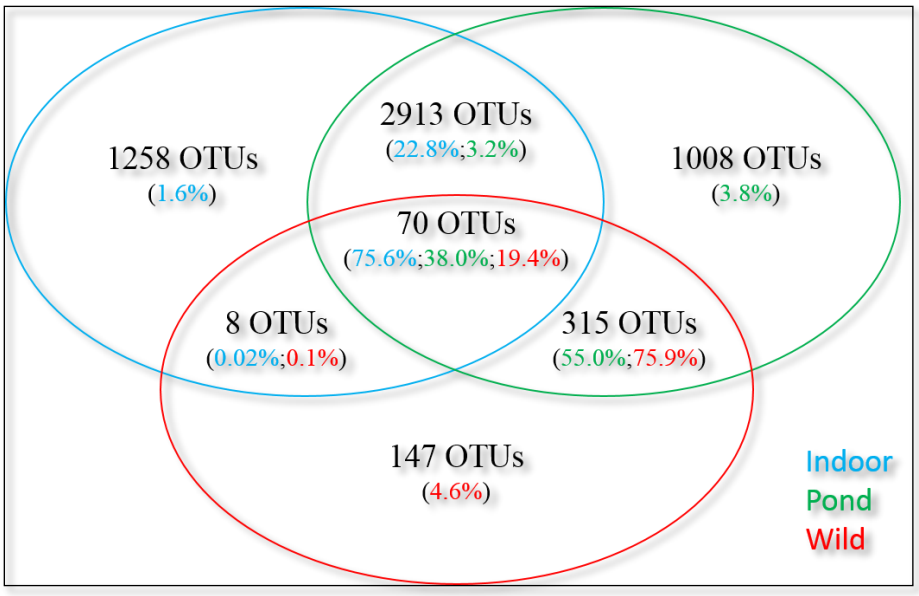

Table 3. Relative abundance (%) of main bacterial taxonomic groups in the intestinal tract of whiteleg shrimp raised under two different production systems and from a wild population.

Taxonomic affiliation	Indoor[a]	Ponds[a]	Wild
Proteobacteria[#]	88.6 ± 3.8	51.8 ± 5.4	60.0
Rhodobacteraceae[#]	84.4 ± 3.8	5.1 ± 5.1	2.7
Vibrionaceae[#]	0.03 ± 0.01	44.8 ± 5.9	53.5
Other Proteobacteria[#]	4.2 ± 0.9	1.8 ± 0.7	3.8
Firmicutes[#]	0.7 ± 0.1	36.0 ± 5.9	18.7
Fusobacteria[#]	0.0 ± 0.0	7.9 ± 2.4	3.2
Bacteroidetes	2.2 ± 2.0	1.6 ± 0.5	2.2
Candidatus Saccharibacteria	2.7 ± 2.2	0.5 ± 0.05	0.0
Cyanobacteria[#]	0.001 ± 0.001	1.6 ± 0.8	7.6
Actinobacteria[#]	3.0 ± 1.1	0.06 ± 0.05	0.2
Other phylum	1.6 ± 1.2	0.3 ± 0.2	6.0
Unclassified bacteria	1.2 ± 0.3	0.8 ± 0.2	2.3

[a] Values shown represent mean and standard error of the mean, respectively. [#] Means of indoor and ponds samples were statistically different ($P < 0.05$).

4.2. Comparative Alpha and Beta Diversity Analyses

To further explore differences in bacterial community composition between the shrimp farming environments investigated, we conducted an alpha diversity analysis. Gut bacterial communities of indoor and pond-farmed shrimp were not found to be statistically different in terms of number of observed OTUs or diversity indices such as chao1, ace, Shannon or Simpson (Table 4). However, PCoA using Bray-Curtis distances based on OTU compositional dissimilarity showed clear differences between the intestinal bacterial communities of shrimp raised indoor and those from pond-farmed shrimp (Figure 2). With the exception of the Ob-1 sample, there was a clear separation between the respective sets of samples. Similarly, hierarchical cluster analysis also

indicated that indoor and pond samples (with the exception of the Ob-1 sample) grouped separately based on their OTU composition (Figure 3).

Table 4. Alpha diversity indices and coverage from gut bacterial communities of whiteleg shrimp raised under two different production systems.

Index	Indoor	Ponds	*P* value	Wild
Observed OTUs	252 ± 27	177 ± 30	0.0746	422
Ace	1488 ± 327	788 ± 258	0.1082	660
Chao1	724 ± 119	462 ± 129	0.1441	609
Shannon	2.21 ± 0.12	2.13 ± 0.13	0.6403	3.00
Simpson	0.28 ± 0.02	0.28 ± 0.03	0.9682	0.23
Coverage (%)	96.7 ± 0.4	97.9 ± 0.5	0.0685	96.1

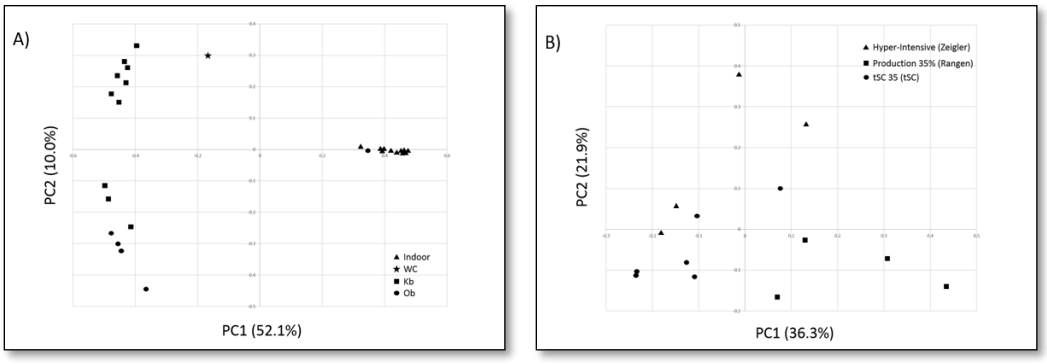

Figure 2. Comparison of intestinal bacterial communities from whiteleg shrimp using Principle Coordinate Analysis (PCoA). A) Comparative analysis between shrimps raised under two different production systems and from one wild population. B) Comparison amongst samples from white-leg shrimp raised in an indoor system under three different diets. The x and y axes correspond to Principal Components 1 (PC1) and 2 (PC2), which explained the highest level of variation.

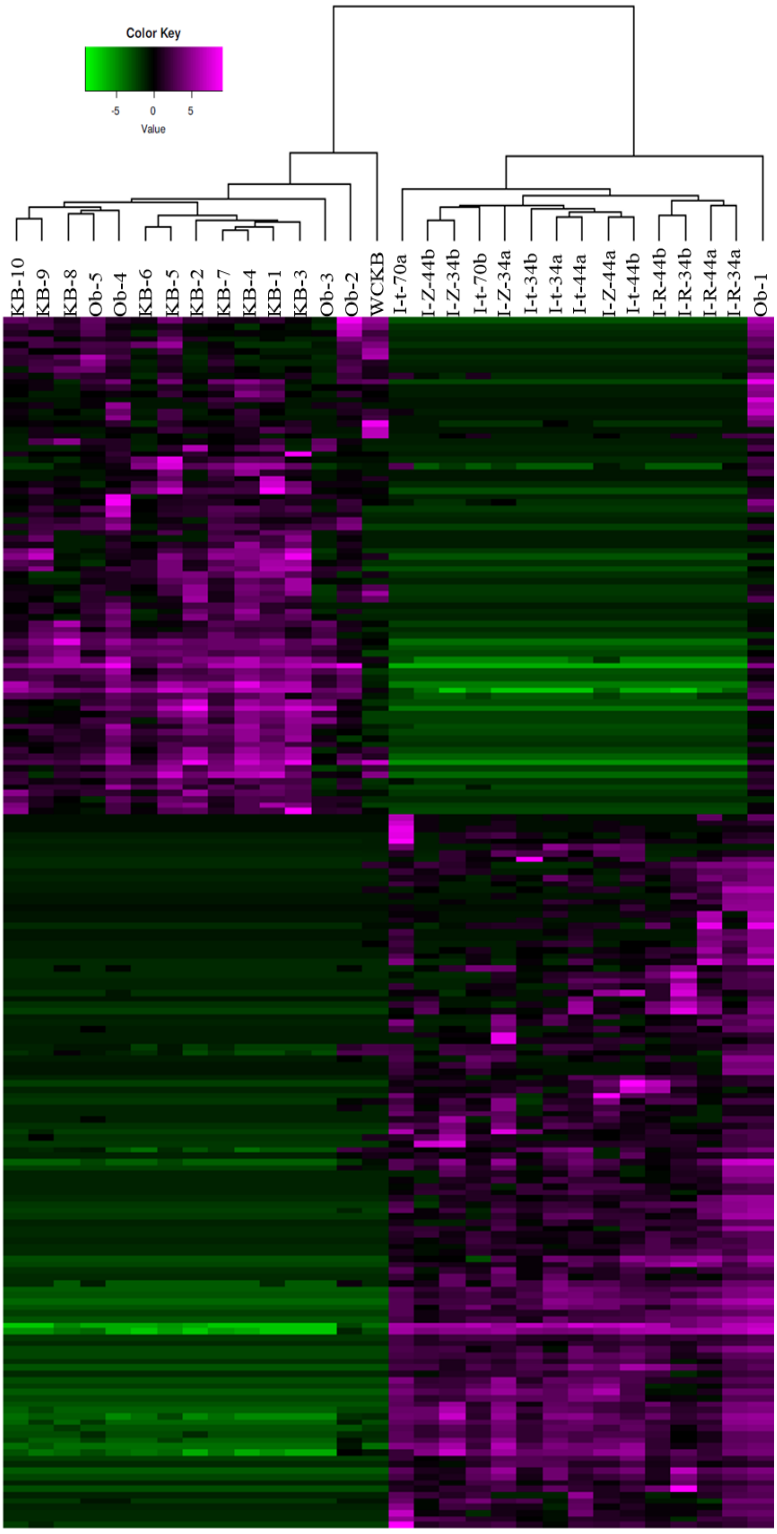

Figure 3. Hierarchical cluster analysis based on the 200 most abundant OTUs from the intestinal bacterial communities of white-leg shrimp.

4.3. Comparative Analysis of Prominent OTUs

To gain further insight, the most abundant bacterial OTUs identified in this study were investigated in more detail. As expected from the taxonomic composition and beta diversity analyses presented above, the profile of main bacterial OTUs from the gut of indoor-raised shrimp was very different from pond-farmed shrimp, with the abundance of 11 of the 12 most prominent OTUs found to be statistically different between the two environments (Table 5). The four prominent OTUs of indoor-raised shrimp (SD_Shr-00001, SD_Shr-00002, SD_Shr-00006, and SD_Shr-00009) were affiliated to the family Rhodobacteraceae (phylum Proteobacteria), together representing on average 72.2% of identified bacteria in these samples, in contrast to 4.1% in pond-raised shrimp. Six other main OTUs were dominant in pond-raised shrimp, and they were affiliated to Vibrionaceae of the phylum Proteobacteria (SD_Shr-00004 and SD_Shr-00005), Firmicutes (SD_Shr-00003 and SD_Shr-00008), as well as Fusobacteria (SD_Shr-00007 and SD_Shr-00015). Interestingly, five of the main pond OTUs were not detected in indoor-raised shrimp. Wild-caught shrimp showed a distinct OTU profile as well, with predominance of SD_Shr-00005 and SD_Shr-00046, which were found in much higher abundance in this sample compared to either indoor or pond-raised shrimp. All Proteobacteria-affiliated main OTUs were found to be very closely related to a known species, with sequence identity values ranging between 98.5% and 99.6%, indicating that each may have represented a strain of its respective closest relative. Conversely, two of the Firmicutes-affiliated OTUs that were prominent in pond-raised shrimp (SD_Shr-00003 and SD_Shr-00008) showed very limited identity to their respective closest

validated taxon, indicating that they may have corresponded to members of a bacterial phylogenetic lineage that is yet to be characterized.

Table 5. Relative abundance (%) of main Operational Taxonomic Units (OTUs) in the intestinal tract of whiteleg shrimp raised under two different production systems and from a wild population.

OTUs	Indoor[a]	Ponds[a]	Wild	Closest valid taxon (id%)
Proteobacteria				
SD_Shr-00001[#]	37.9 ± 4.9	1.5 ± 1.5	0.07	*Phaeobacter piscinae* (98.5%)
SD_Shr-00002[#]	23.8 ± 3.1	2.3 ± 2.2	0.2	*Thalassobacter stenotrophicus* (98.5%)
SD_Shr-00004[#]	0.02 ± 0.007	26.8 ± 4.6	1.8	*Vibrio alginolyticus* (99.1%)
SD_Shr-00005[#]	0.0 ± 0.0	6.4 ± 2.3	46.4	*Photobacterium damselae* (99.1%)
SD_Shr-00006[#]	7.8 ± 2.1	0.2 ± 0.1	0.4	*Ruegeria profundi* (99.4%)
SD_Shr-00009[#]	2.7 ± 0.8	0.1 ± 0.1	0.09	*Roseovarius pacificus* (99.6%)
Firmicutes				
SD_Shr-00003[#]	0.0 ± 0.0	28.2 ± 6.2	0.5	*Oceanobacillus iheyensis* (80.9%)
SD_Shr-00008[#]	0.0 ± 0.0	3.5 ± 1.1	0.09	*Oceanobacillus iheyensis* (80.6%)
SD_Shr-00046[#]	0.003 ± 0.001	0.02 ± 0.004	12.2	*Romboutsia lituseburensis* (98.2%)
Fusobacteria				
SD_Shr-00007[#]	0.0 ± 0.0	4.4 ± 1.7	0.1	*Propionigenium maris* (96.4%)
SD_Shr-00015[#]	0.0 ± 0.0	1.7 ± 0.6	3.0	*Propionigenium modestum* (91.3%)
Cyanobacteria				
SD_Shr-00021	0.0 ± 0.0	0.8 ± 0.5	2.5	*Gloeobacter kilaueensis* (86.2%)

[a] Values shown represent mean and standard error of the mean, respectively. [#] Means of indoor and ponds samples were statistically different ($P < 0.05$).

5. Discussion

In this study, the intestinal bacterial communities of whiteleg shrimp raised under different aquaculture production systems were investigated. Considering the paucity of data available from indoor-raised shrimp, samples were collected from animals fed different diets at various stages of their development in an effort to be representative of potential variability in bacterial profiles. Remarkably, indoor-raised samples showed very homogenous gut bacterial taxonomic composition and community structures regardless of diet and growth stage, as best shown by PCoA analysis (Figure 2). While gut bacterial profiles from pond-raised shrimp were not as closely related to each other as indoor samples, they were consistent as a group and very distinct from those of indoor-raised shrimp. Notably, 11 of the most abundant OTUs were significantly different between the two types of samples, suggesting an effect of production systems on the intestinal bacterial communities of the whiteleg shrimp.

In terms of phylogeny, genetics and metabolic potential, bacteria typically represent the most diverse group of microorganisms in animal gut environments. The relationship between a host and its symbiotic bacteria is the result of co-evolution between the two entities, as mutually beneficial adaptations are selected to favor their association (E. Li et al., 2018). The development of gut microbial communities in hatchling or neonatal animals involves successive waves of colonization and succession, concurrent with the development, maturation, and food habits of their host. The development of the intestinal microbiome in whiteleg shrimp starts during the fifth nauplius stage, as movement of fluid through the gut is initiated by the anal pore, later followed by major changes at mysis and early post larval stages that occur as a result of

feeding on invertebrates such as Artemia and Rotifera spp. after hatching (E. Li et al., 2018). Gut bacterial communities during all developmental stages consist primarily of Proteobacteria, Bacteroidetes and Actinobacteria (Huang et al., 2016), with representation of each group fluctuating in response to diet changes and development of their host. Other factors, such as salinity, stress, host immune response, exposure to antibiotics, and environmental conditions can affect the composition of gut microbial communities (Cornejo-Granados et al., 2017). Consequentially, these factors have a great influence on the ability of gut microbiomes to contribute to the health and nutrition of their host prior and during the productive stages of their life cycle. While many of the mechanisms involved remain to be resolved, a wide body of research focused on humans and animal models has indicated that the type of microorganisms that a young animal is exposed to can affect the composition of its microbiome as it matures.

In natural aquatic systems, shrimp are exposed to microorganisms from water and sediments which provide a pool or source of potential symbionts that can colonize their intestinal tract at various stages of their development (Cornejo-Granados et al., 2017). Indeed, at least ninety genera have been found to be shared amongst pond sediments, pond water and the shrimp intestinal tract, with the most similarities found between sediment and gut profiles (Sun et al., 2016). Because Lactobacillus, Streptococcus and Bacillus are the most abundant shared genera in the shrimp intestinal tract (1.0%, 0.93% and 0.37%, respectively) (E. Li et al., 2018), and that they are already used as probiotics in humans and other food animals, they have been a major source of probiotics in the shrimp aquaculture industry. While their abundance tends to be low (<1%) in the shrimp intestinal environment, their use as probiotics has been found to be beneficial to the

immunity of the host and its efficiency in digesting commercial diets (Huang et al., 2016). In this current study, however, they were found at much lower levels, with Lactobacillus identified in only one pond shrimp sample (0.01%), Streptococcus in three indoor shrimp samples and one pond shrimp sample (0.02% or less), and Bacillus not detected in any of the shrimp samples analyzed. However, it is possible that these probiotic species could affect the composition of bacterial communities in the gut of indoor-raised shrimp even if they do not appear to become established in that environment. As suggested elsewhere (E. Li et al., 2018), perhaps probiotic formulations for shrimp production should include beneficial bacterial strains that are native to the shrimp gut.

A number of animal health problems in shrimp can be caused by poor nutrition, as it leads to undeveloped or weak individuals that become more susceptible to disease (Xiong et al., 2017). The risk for such nutrition problems may be higher with commercial dietary formulations, since the industry is transitioning from fish meal and oils to plant-based ingredients in order to reduce operating costs (E. Li et al., 2018). Even though the shrimp sampled from indoor production tanks were sourced from distinct tank populations, had independent water sources and management, and were each fed one of three dietary treatments, their gut bacterial profiles were remarkably similar (Figure 1 and Figure 2). Because the indoor diets were formulated using varying combinations of animal and plant-derived protein sources, these results would indicate that while shrimp gut bacterial profiles are highly influenced by dietary ingredients, as shown by the comparison between indoor-raised and pond-raised shrimp in this report, bacterial composition may not be as sensitive to variations in formulations with the same set of

ingredients. Plant-derived protein sources in commercial shrimp diets tend to also include polysaccharides and anti-nutritional factors, which the shrimp intestinal tract and natural microbiome have not evolved to digest effectively. Interestingly, while members of the phylum Bacteroidetes are typically considered to be the main utilizers of plant polysaccharides in most gut environments, their abundance was not found to vary significantly amongst the different shrimp production environments investigated (Table 3). While future investigations will be required to elucidate the mechanisms involved, perhaps bacteria from other phylum allow farmed shrimp to effectively use plant-derived protein ingredients. Likely candidates would include SD_Shr-00001, SD_Shr-00002, SD_Shr-00006, and SD_Shr-00009, which were OTUs affiliated to Rhodobacteraceae that were much more abundant in indoor-raised shrimp compared to pond or wild-caught populations. Intriguingly, Firmicutes have been reported in higher abundance in the gut of whiteleg shrimp fed corn starch compared to shrimp fed glucose and sucrose (Qiao et al., 2017).

In addition to diet, exposure to stress or other perturbations can disrupt composition of gut microbiomes resulting in a state of dysbiosis which can provide an opportunity for pathogenic bacteria to thrive, leading to disease (Xiong et al., 2017). During the shrimp production cycle, stress can be induced from overcrowding or changes in water salinity and quality (E. Li et al., 2018). While their effect may be undetectable under normal farming conditions, opportunistic pathogens are thought to be ubiquitous in the microbiome of healthy individuals (Huang et al., 2016). The main bacterial pathogens that can infect shrimp include members of the genera Pseudomonas, Flavobacterium, Escherichia, Aeromonas, Vibrio, Rickettsia, Shewanella and Desulfovibrio (E. Li et al.,

2018). Amongst these, Vibrio-affiliated sequences were by far the most abundant in this study, particularly OTU SD_Shr-00004, with a mean abundance of 26.8% in pond-samples (Table 4). In contrast, no sequences affiliated to Flavobacterium, Escherichia, Aeromonas or Rickettsia were identified amongst the 30 samples analyzed, while sequences for Desulfovibrio (0.02% or less in three pond samples, 0.3% in the wild-caught sample), Shewanella (0.003% or less in two pond samples) and Pseudomonas (one indoor and one pond sample at 0.003% or less, respectively) were only found at very low representation. Notably, SD_Shr-00004 was very closely related to Vibrio alginolyticus, an opportunistic pathogen that can cause disease under stress conditions (Chen, Chen, Tseng, Lin, & Huang, 2015; Santhiya & Sivasankar, 2015). V. alginolyticus, as well as V. parahaemolyticus, V. harveyi, V. vulnificus and V. damsel, are commonly present in aquaculture production systems (Chen et al., 2015; Santhiya & Sivasankar, 2015). While the levels of SD_Shr-00004 were far lower in indoor-raised shrimp compared to pond-farmed shrimp, its detectable presence in animals raised in a controlled environment suggests it may be a normal resident in the gut of shrimp. Perhaps much higher abundance, as observed in pond shrimp, could be indicative of a state of dysbiosis. However, since the captured shrimp with high levels of SD_Shr-00004 in this study did not show signs or obvious symptoms of disease, perhaps much higher proliferation of SD_Shr-00004 would be necessary to reach a diseased state or the OTU identified in this study is not a strain of V. alginolyticus. Interestingly, Actinobacteria which have recently been reported as a key bacterial phylum in the gut of whiteleg shrimp that allows growth under low salinity conditions and prevents uncontrolled growth of pathogens (E. Li et al.,

2018) was on average 50X more abundant in indoor farmed shrimp compared to pond-raised shrimp in this current study.

Together, the results presented in this report indicate that aquaculture practices greatly influence the intestinal bacterial profile of whiteleg shrimp, and further suggest the bacterial communities of this economically important crustacean could in the future be effectively manipulated using diet composition or environmental factors such as water conditions. While future research is necessary to elucidate the mechanisms involved, it also opens the possibility that the same potential for microbiome modulation could exist in other economically important shrimp species, such as the Giant tiger prawn /Asian tiger shrimp (Penaeus monodon), Chinese white shrimp /Oriental shrimp /Fleshy prawn (Fenneropenaeus chinensis), Brown tiger prawn (Penaeus penicillatus), and Banana prawn (Penaeus merguiensis).

CHAPTER TWO

Host genetics is an important determinant of gut bacterial community composition in aquaculture-raised Pacific Whiteleg shrimp *Litopenaeus vannamei*

1. Abstract

This study presents potential effects of genetic background and the use of probiotics on the gut bacterial composition of Pacific whiteleg shrimp (*Litopenaeus vannamei*) grown in an indoor aquaculture facility. The strains investigated were Shrimp Improvement Systems (SIS, Islamorada, Florida, USA), a strain genetically selected for disease resistance, and an Oceanic Institute (OI, Oahu, Hawaii, USA) strain, selected for growth performance. BioWish 3P (BioWish Technologies, Cincinnati, USA) was the selected probiotic. The study consisted of two separate trials, where all shrimp were raised under standard industry conditions and fed the same diet. Shrimp were stocked in 2,920 L production tanks at a density of 200/m3 and acclimated for 14 days. After the acclimation period, triplicate tanks were supplemented daily for a duration of 28 days with probiotics, while three other tanks did not receive any treatment (controls). During the 28-day trial period, there was no statistically supported difference ($P > 0.05$) in either performance or health status as a result of genetic background or probiotic treatment. However, differences in gut bacterial composition, as assessed by high throughput sequencing of amplicons generated from the V1-V3 region of the bacterial 16S rRNA gene, were observed. The relative abundance means of six major Operational Taxonomic Units (OTUs) were found to vary significantly across experimental groups ($P < 0.05$), all showing distinct patterns specific to genetic lines. Notably, SD_Shr-00006 was in highest abundance in d43 SIS samples, with levels greater than d71 samples of the same genetic

line or any of the OI shrimp samples. OTUs for SD_Shr-00098 displayed a similar type of profile, but with highest abundance in the OI genetic line and lowest in the SIS shrimp. SD_Shr-00004 showed an opposite profile, with highest abundance ($P < 0.05$) in the SIS d71 samples and lowest in the SIS d43 samples. Together, these results support that host genetic background is an important determinant of gut bacterial composition in aquaculture-raised whiteleg shrimp and indicate that development of strategies to manipulate the microbiome of this important seafood will likely need to be customized depending on the genetic line.

2. Introduction

Shrimp represent the most valuable seafood in the world, with a hold on 78% of the seafood monetary market (SOFIA, 2018). This industry has managed to grow despite stagnant yields in wild-caught harvests through a substantial increase in aquaculture production. Indeed, 55% percent of the annual global shrimp supply in 2018 was produced through commercial farming (Gaille, 2018), indicating that aquaculture has the capacity to provide consumers with a consistent and reliable supply of product (Ellis et al., 2011). Shrimp farming has shown great potential for high productivity at reduced costs. Notably, aquaculture-raised shrimp have shown twice the growth rates of wild stocks, indicating great potential to further increase production (Greenberg, 2012). However, periodic disease outbreaks and continued animal health management problems have become a great concern to potential investors, and consequently to the future of aquaculture. In order to mitigate losses in production due to disease, some major producers have opted to generate greater quantities of smaller-sized product by

harvesting shrimp at an earlier stage, before significant losses can occur. However, flooding the markets with such lower value product can only be viable in the short term, as it may impact consumer demand (SOFIA, 2018). As the market for shrimp continues to increase worldwide, the aquaculture industry needs to continue developing innovative strategies to take advantage of market growth opportunities.

Two main types of production systems are generally used in shrimp aquaculture. Outdoor systems, which typically consist of ponds that rely on natural saltwater sources for maintaining optimal growth conditions, offer a low-cost opportunity for shrimp aquaculture. In contrast, the more costly indoor facilities allow tighter biosecurity control, safer products and reduced environmental footprint as they use recirculating water systems with limited exposure to natural environments (Jobling, 2013). The most widely used species worldwide for both outdoor and indoor production systems is the whiteleg shrimp (*Litopenaeus vannamei*), also known as Pacific white shrimp or king prawn, as it expresses a number of desirable traits, such as tolerance to a wide range of salinities and temperatures (Cheng et al., 2006; E.-C. Li et al., 2017). Overall, the development of shrimp genetic lines has focused mainly on increased growth and higher disease resistance, as these two traits are of major importance for aquaculture production. However, because they are mutually exclusive in inheritance (Andriantahina et al., 2013), breeding programs typically focus on one of these traits depending on the production system. Because of greater risks of exposure to opportunistic pathogens, disease resistance is of higher importance for outdoor systems, while genetic line development for bio-secure indoor systems has focused on increasing shrimp growth since exposure to environmental pathogens is greatly reduced (Andriantahina et al., 2013). The use of

rapidly growing shrimp with reduced disease resistance comes at a risk, however, as systemic stressors can promote the growth of ubiquitously present *Pseudomonas* or *Vibrio* species that can colonize the shrimp gut and cause disease (E. Li et al., 2018). One possible strategy to mitigate risk of disease in susceptible genetic lines would be to promote health through microbial management (Jobling, 2013). Considering the overarching importance of the gut microbiome in animal health and nutrition (Cornejo-Granados et al., 2017), manipulating beneficial gut microbial communities could potentially improve aquaculture shrimp resistance to pathogens without prophylumctic use of antibiotics.

Research to date has found that the gut bacterial profile of healthy shrimp consists primarily of Proteobacteria (Califano et al., 2017). This is consistent with reported profiles in marine fish, but in stark contrast to the microbiome of terrestrial animals in which Firmicutes and Bacteroides are typically dominant (Xiong et al., 2017). Indeed, these latter phylum have so far been found to be minor components of the shrimp gut microbiome, and their abundance appears to be highly dependent on local environmental conditions and diet composition (E. Li et al., 2018). While great strides have so far been made towards defining the composition of gut bacterial communities in healthy shrimp, there is a critical need to determine how composition is impacted by production parameters. Based on a wide body of research literature on human and terrestrial animal microbiomes, host genetics is likely an important factor affecting the composition of gut symbionts in shrimp. A number of management practices also have the potential to influence gut bacterial community profiles, but there is limited in-depth knowledge to date on the effect of aquaculture practices on the shrimp gut microbiome (Cornejo-

Granados et al., 2017). For instance, the biosafety measures implemented to prevent pathogen outbreaks, such as water and feed sterilization, may also inadvertently affect gut colonization of indoor raised shrimp as these procedures limit exposure to beneficial bacteria found in natural environments (Cornejo-Granados et al., 2017). Absence of certain microorganisms during early events of microbiome development in shrimp raised indoor could possibly impact future performance, productivity or disease resistance (Huang et al., 2016). Other factors that can affect gut bacterial community profiles include water chemistry and diet composition. For instance, feeds being offered in aquaculture are trending towards formulations with increased proportions of less expensive ingredients, such as plant proteins, fiber, and carbohydrates, which are not natural components of shrimp diets (Huang et al., 2016). Finally, the use of probiotics can also possibly affect the composition of gut bacterial communities. Probiotics have historically been used for competitive exclusion of pathogens during stress events in pond systems (Samocha et al., 2004), but they also represent a potential means of transitioning gut microbiomes for improved gut health and digestion of plant-based feed ingredients.

In light of the critical need to gain further insight on the influence of host genetics and management practices on the shrimp microbiome, the research presented in this report aimed to investigate the possible impact of host genetic and probiotics use on modulating the composition of the whiteleg shrimp microbiome. To this end, the gut bacterial profiles of whiteleg shrimp raised in an indoor facility with the same diet were determined from two different genetic lines, with or without supplementation with commercial probiotics. We found that the abundance profile of specific bacterial taxa and Operational Taxonomic Groups (OTUs) varied significantly between genetic lines.

3. Materials and Methods

3.1. Shrimp Aquaculture Production System

The research described in this report was conducted at the trū Shrimp Innovation Center (330 3rd Street, Balaton, MN, USA; 44.2°N 95.8°W), a research campus designed for the development of innovative indoor aquaculture techniques for shrimp production. Six polyethylene aquaculture production tanks (2.75 m x 6.7 m x 0.4 m) were used for this study, each controlled by an independent system to maintain temperature (28.0 ± 1.0oC), provide water circulation (0.075-0.15 linear meters per second) and aeration (while rates varied according to production phase, dissolved oxygen levels were maintained at 4.5 mg/L or higher).

Each tank contained approximately 2,920 L of saltwater, with an average depth of 0.3 m. Salt water was prepared by dissolving Crystal Sea Marinemix (Marine Enterprises International, LLC., Baltimore, Maryland) with reverse osmosis-produced water at a final concentration of 28g / L. Salinity was monitored and maintained at 28 ppt by adding approximately 58 L/day of reverse osmosis water to replace losses due to evaporation. Tank pH was kept at 7.7 ± 0.5 and alkalinity maintained at 150 - 300 ppm, with adjustments made by supplementation with sodium carbonate (9mg /L of tank water for 0.5 unit reduction in pH below 7.4) or sodium bicarbonate (14 mg/L of tank water for 10 unit reduction in alkalinity), respectively. Fresh reverse osmosis water was added as needed to maintain tank depth.

Water from each tank was biologically filtered using separate floating bead bed bioreactors at a flow rate of 15 ± 0.5 L / m. Each bioreactor had a capacity of 0.015 m3 and

consisted of a standard bio-medium of spherical beads, made of low-density polyethylene with a diameter of 1/8 inch and a surface area of 1100-1200 m2/m3 (catalog# BEADSFT3 Aquaculture Systems Technologies). Bioreactors were set up five days prior to the addition of shrimp in the production tanks by inoculation with 366mL of a commercial bacterial stock (catalog# 75080590; FritzZyme Industries, Dallas, TX, USA), followed by daily supplementation with 8g of ammonium chloride and 5g of sodium nitrite to promote development of bacterial biofilms.

Tanks were also inoculated with autotrophic bacteria five days prior to stocking using a commercial product consisting of Nitrobacter and Nitrosococcus species (0.135 mL stock per L of tank water; FritzZyme Industries, Dallas, TX, USA). Additional autotroph dosages were provided on trial days 1, 2, 12, 14, and 16. Further supplementations were provided when the total ammonia nitrogen (TAN) and/or nitrite levels exceeded concentrations of 5 mg/L and 2.5 mg/L, respectively.

Once bioreactor inoculation was completed, tanks were stocked with post-larval stage 12 shrimp, which were grown for 30 days. Following this period, all shrimp were removed from their respective tanks, pooled, then randomly redistributed at a stocking density of 2,000 shrimp / tank (0.85g ± 0.1g). The stocking density (200 shrimp / m3, with a weight-based density of 0.175 ± 0.5 kg/m3) after redistribution was consistent with super-intensive production systems and was predicted to yield a final harvest density of 1.54 ± 0.5 kg/m3. Shrimp were then allowed to acclimate for 14 days before probiotic treatment (see below). The extruded feed (proprietary formulation) was manufactured by Prairie AquaTech (Brookings, SD, USA), with pellet diameters adjusted for shrimp growth, increasing from 1.8 to 2.4 mm during the course of the study. Feed ingredients included animal (fish and

poultry meals), as well as plant products (soy and wheat meals); a proximate composition analysis of representative feed samples (Midwest Laboratories, Omaha, NE, USA) conducted prior to the experiment is presented in Table 1.

Table 1. Proximate analysis for proprietary diet fed to all research tanks.

Analyzed Nutrients	Units	Observed Value
Moisture	%	6.85
Dry Matter	%	93.15
Protein (crude)	%	37.50
Fat (crude)	%	9.54
Fiber (crude)	%	1.80
Ash	%	11.40
Digestible Energy	Mcal/lbs	1.55
Total Digestible Nutrients	%	77.10
Metabolizable Energy	Mcal/lbs	1.36
Net Energy (gain)	Mcal/lbs	0.56
Net Energy (lactation)	Mcal/lbs	0.81
Net Energy (maint.)	Mcal/lbs	0.84

3.2. Experimental Design

The study consisted of two separate trials, with each trial conducted once using shrimp from a single genetic line. Two genetic lines of *Litopenaeus vannamei* were used: Shrimp Improvement Systems (SIS, Islamorada, Florida, USA), selected for disease

resistance, and Oceanic Institute (OI, Oahu, Hawaii, USA), selected for growth. During each trial, three replicate tanks were supplemented with a commercial probiotic, while the remaining three tanks did not receive any supplementation (controls). Treatment tanks received a daily dose of the probiotic BioWish 3P (BioWish Technologies, Cincinnati, USA) for 28 days, at a rate of 0.73g / day. The probiotic was provided with the feed, which was offered over a 24h period. The BioWish 3P product contained *Pediococcus acidilactici* ($\geq 1 \times 10^8$ cfu/g), *Pediococcus pentosaceus* ($\geq 1 \times 10^8$ cfu/g), *Lactobacillus plantarum* ($\geq 1 \times 10^8$ cfu/g), and *Bacillus subtilis* ($\geq 1 \times 10^7$ cfu/g).

3.3. Analytical Methods

3.3.1. Monitoring of Water Quality

Water chemistry testing was performed using industry validated methods (TAN method 8155 DR800, alkalinity method 10239 TNTplus, nitrite method 10019 DR800, LR, Test 'N Tube, and nitrate method 8039 Cadmium Reduction). A Hach spectrophotometer (Hach Company, Loveland, Colorado, USA) was used for measurements. Total ammonia nitrogen (TAN), calculated un-ionized ammonia concentrations, as well as alkalinity were monitored daily. Nitrite and nitrate levels were measured three times every week (Mondays, Wednesdays, and Fridays). Dissolved oxygen, pH, temperature, and salinity were monitored daily using a YSI Professional Plus handheld multi-parameter meter (YSI Incorporated, Yellow Springs, Ohio, USA).

3.3.2 Shrimp Performance and Health Assessments

Initial exams were performed from five randomly selected representative shrimp prior to stocking. Each selected individual was patted dry, weighted, then measured for total length (telson to the tip of the tail) and antenna length (head to the antenna tip). Body surface was then examined for necrosis or signs of injury, and gut fullness was assessed by examining the intestinal tract through the muscle wall, using a scale from 0 (100% full) to 4 (0-24% full). After pulling back the carapace, gills were then evaluated for presence of necrosis and / or debris using a scale from 0 (tissue completely clear of necrosis or debris) to 4 (15+ areas of necrosis and / or debris in a single viewing field). Finally, the hepatopancreas was dissected, weighed and examined for coloration and appearance, which were evaluated using a scale from 0 (no deformities, healthy organ) to 4 (16+ areas of severe tubular deformation, chronic ailment) (A Bell & V Lightner, 1988).

During the trial, shrimp were sampled at seven-day intervals to monitor growth, performance and health. A total of 90 shrimp were netted, removed from the tank, then weighed individually. All but five randomly selected shrimp were returned to the production tank. Animal health exams as described above were then performed on the five selected shrimp.

The following health indices were calculated from the health exam data:

The mean condition factor (MCF), was determined with the equation: $MCF = \frac{100*(Shrimp\ Weight\ (g))}{(Shrimp\ Length\ (cm))^3}$ (Abohweyere & Williams, 2008). An MCF of less than 0.8 was indicative of low girth and poor health.

The hepatosomatic index (HSI) was calculated with the equation: $HSI = \frac{Hepatopancreas\ Weight\ (g)}{Shrimp\ Weight\ (g)}$ (Chellappa, Huntingford, Strang, & Thomson, 1995). HSI is an

assessment of a shrimp's potential energy reserves; a value of 0.03 or lower indicated poor nutrient availability or absorption, while a value of 0.09 or above was a sign of possible systemic pathogenic infection.

The shrimp to antenna length ratio (SAR) was obtained with the following equation: $SAR = \frac{Average\ Antenna\ Length\ (cm)}{Shrimp\ Length\ (cm)}$. An SAR score of 0.5 or lower was indicative of stress that could result in failure to thrive or death.

3.3.3. Microbiological Analyses of Tank Water

Tank water samples were tested prior to stocking, then later on a weekly basis during the trial, to assess microbial populations for total heterotrophs, pathogenic *Vibrio,* as well as non-pathogenic *Vibrio*. Approximately 50 mL of tank water were collected with a sterile serological pipette and stored in a sterile 50 mL screw cap conical tube until analyzed by a commercial diagnostic laboratory (Research Technology Innovation Laboratory (RTI), Brookings, SD, USA). For diagnosis, serial dilutions were prepared to determine total heterotrophic counts by plating on marine-agar medium (Buck & Cleverdon, 1960) (Patrick, 1978), as well as total *Vibrio* counts by plating on thiosulfate-citrate-bile salts-sucrose-agar medium. Potential pathogenic *Vibrio* colonies were distinguished from non-pathogenic by their color response on the selective media (Baron, Peterson, & Finegold, 1994). *Pediococcus* counts were determined by plating on De Man, Rogosa and Sharpe agar (MRS).

3.4. Microbial DNA Isolation and PCR Amplification

Shrimp gut samples were analyzed for bacterial composition on days 43 (prior to probiotic treatment, pre-treatment control), 57 (14 days probiotics treatment), and 71 (28 days probiotics treatment). Intestinal tissue was harvested from each animal using the following procedure. The telson was removed distal to the sixth abdominal segment with scissors, then the posterior end of the carapace was lifted to expose the hepatopancreas and the proximal end of the gut. The intestine was then excised with sterile tweezers starting at the hepatopancreas on through to the hind gut. Each sample consisted of intestinal tissue pooled from five individual shrimp from the same population to ensure sufficient material was available for DNA extraction. All harvested intestinal tissue samples were stored with no preservative at -20°C until DNA extraction.

Microbial DNA was isolated from gut samples using the repeated bead beating plus column method, as described by Yu and Morrison (Yu & Morrison, 2004). The V1-V3 region of the bacterial 16S rRNA gene was PCR-amplified using the 27F forward (Edwards et al., 1989) and 519R reverse (Lane et al., 1985) primer pair. PCR reactions were performed with the Phusion Taq DNA polymerase (Thermo Scientific, Waltham, MA, USA) under the following conditions: hot start (4 min, 98 °C), followed by 35 cycles of denaturation (10s, 98 °C), annealing (30s, 50 °C) and extension (30 s, 72 °C), then ending with a final extension period (10 min, 72 °C). PCR products were separated by agarose gel electrophoresis, and amplicons of the expected size (~500bp) were excised for gel purification using the QiaexII Gel extraction kit (Qiagen, Hilden, Germany). For each sample, approximately 400 ng of amplified DNA were submitted to Molecular

Research DNA (MRDNA, Shallowater, TX, USA) for sequencing with the Illumina MiSeq 2X300 platform to generate overlapping paired-end reads.

3.5. Computational Analysis of PCR Generated 16S rRNA Amplicon Sequences

Unless specified, sequence data analysis was performed using custom written Perl scripts (available upon request). Raw bacterial 16S rRNA gene V1-V3 amplicon sequences were provided by Molecular Research DNA as assembled contigs from overlapping MiSeq 2x300 paired-end reads from the same flow cell clusters. Reads were then selected to meet the following criteria: presence of both intact 27F (forward) and 519R (reverse) primer nucleotide sequences, length between 400 and 580 nt, and a minimal quality threshold of no more than 1% of nucleotides with a Phred quality score lower than 15.

Following quality screens, sequence reads were aligned, then clustered into Operational Taxonomic Units (OTUs) at a genetic distance cutoff of 5% sequence dissimilarity (St-Pierre & Wright, 2015). While 3% is the most commonly used clustering cutoff for 16S rRNA, it was originally recommended for full length sequences, and may not be suitable for the analysis of specific sub-regions since nucleotide sequence variability is not constant across the entire length of the 16S rRNA gene. In this context, if 3% is a commonly accepted clustering cutoff for V4 or V4-V5 regions, which are the least variable of the hypervariable regions, then a higher cutoff should be used for the V1-V3 region, since V1 is the most variable region of the 16S rRNA gene. OTUs were screened for DNA sequence artifacts using the following methods. Chimeric sequences were first identified with the chimera.uchime and chimera.slayer commands from the

MOTHUR open source software package (Schloss et al., 2009). Secondly, the integrity of the 5’ and 3’ ends of OTUs was evaluated using a database alignment search-based approach. When compared to their closest match of equal or longer sequence length from the NCBI nt database, as determined by BLAST (Altschul et al., 1997), OTUs with more than 1% of nucleotides missing from the 5’ or 3’ end of their respective alignments were discarded as artifacts. Single read OTUs were subjected to an additional screen, where only sequences that had a perfect or near perfect match to a sequence in the NCBI nt database were kept for analysis, (i.e. that the alignment had to span the entire sequence of the OTU, and a maximum of 1% of dissimilar nucleotides was tolerated).

After removal of sequence chimeras and artifacts, taxonomic assignment of valid OTUs was determined using a combination of RDP Classifier (Wang et al., 2007) and BLAST (Altschul et al., 1997). The List of Prokaryotic Names with Standing in Nomenclature (LPSN - http://www.bacterio.net) was also consulted for information on valid species belonging to taxa of interest (Parte, 2014).

3.6. Computational Analysis for Microbial Community Diversity

For beta diversity analysis, abundance tables were first filtered by removing taxa found less than two times in 10% of the samples, then a relative abundance table was made. Filtering allowed to visualize high-level patterns in the dataset (Dahan, Jude, Lamendella, Keesing, & Perron, 2018). The data, based on Bray-Curtis distances (Bray & Curtis, 1957), was ordinated by Principal Coordinates Analysis (PCoA) (Clarke & Ainsworth, 1993). The PCoA ordination matrix was generated using the ordinate function

and plot by plot ordination function of the package “phyloseq” in R (R Foundation for Statistical Computing, Vienna, Austria).

3.7. Statistical Analysis

Using R (Version R-3.2.3), ANOVA (command aov) and post hoc Tukey Honest Significant Difference (command TukeyHSD) analyses were performed to compare the abundance of bacterial taxonomic groups and OTUs between different groups of replicate samples, respectively. Means were considered to be significantly different when $P \leq 0.05$.

3.8. Accession Numbers for Next Generation Sequencing Data

Raw sequence data are available from the NCBI Sequence Read Archive under Bioproject PRJNA551222.

4. Results

4.1. Comparative Analysis of Production Parameters and Growth Performance

No statistical differences ($P > 0.05$) were observed in performance (total feed offered, feed:gain, average daily gain or survival) between genetic lines or as a result of probiotic treatment. Similarly, no differences in health indices, i.e. mean condition factor (MCF), shrimp-antenna length ratios (SAR) or hepatosomatic index (HSI), were found across samples.

Water chemistry was monitored and managed throughout the trial. Overall, total ammonia nitrogen (TAN), unionized ammonia (UA), and nitrite were numerically higher in OI production tanks compared to SIS production tanks, but these differences were not

found to be statistically significant ($P > 0.05$; Supplementary data). Similarly, other measured water chemistry parameters (nitrate, nitrite, pH, and alkalinity; see Supplementary data) did not vary significantly during the trial. Microbial quality of production tank water was assessed using culture-based microbiological assays. No significant differences were found for total heterotrophic counts ($P > 0.05$). While pathogenic *Vibrio* were not detected using this method, non-pathogenic *Vibrio* species were found at an average density ranging between $4.10x10^3$ and $5.47x10^3$ CFU/ml ($P > 0.05$), which is within a range consistent with normal operating conditions for indoor shrimp aquaculture production (data not shown). Counts for *Pediococcus* ranged between $2.41x10^2$ and $8.25x10^2$ CFUs/ml across all tanks, with no statistical supported differences between genetic lines or as a result of probiotic treatment ($P > 0.05$, see Supplementary data).

4.2. Comparative Analysis of Gut Bacterial Communities by Taxonomic Composition

To investigate the potential effect of host genetic background and / or probiotic treatment on the composition of gut bacterial communities, an analysis using the 16S rRNA gene as a phylogenetic marker was performed. A total of 609,210 high quality and chimera/artifact-free reads were generated across 36 samples, with an average of 13,569 ± 8,158 reads per sample for the SIS genetic line and 20,276 ± 11,064 reads per sample for the OI genetic line.

Consistent with previous published reports on bacterial communities of the shrimp gut, Proteobacteria was found to be the most abundant phylum in this study, with

means of experimental groups ranging between 43.68 and 80.84% (Table 2). Within Proteobacteria, Vibrionales and Rhodobacterales were the most highly represented orders, together accounting for 92.78 to 99.44% of Proteobacteria in individual samples. While found in lower abundance, the phylum Bacteroidetes (1.14 – 45.98%), Firmicutes (0.42 – 50.13%) and Verrucomicrobia (0.51 – 28.80%) were overall well represented across experimental groups. Other minor phylum, such as Planctomycetes (0.34 – 2.97%) and Saccharibacteria (0.02 – 4.87%), were also identified.

Table 2. Mean relative abundance (%) of main bacterial taxonomic groups in the intestinal tract of whiteleg shrimp from two genetic lines (SIS or OI), in the presence (+) or absence (-) of probiotic treatment, at three different sampling time points (d43, d57 and d71).

Taxonomic group	SIS.43	SIS.57+	SIS.57-	SIS.71+	SIS.71-	OI.43	OI.57+	OI.57-	OI.71+	OI.71-	*P* values*
Proteobacteria	77.21	80.84	43.68	87.22	64.93	62.96	79.70	47.35	73.61	56.43	0.09470
Rhodobacterales	47.41^{bc}	8.79 ^{a}	11.13 ^{a}	3.96 ^{a}	9.63 ^{a}	12.94 ^{a}	18.83 ^{ac}	1.86 ^{a}	4.43 ^{a}	6.21 ^{a}	0.00032
Vibrionales	27.93	71.60	31.95	82.70	54.44	47.76	59.04	45.01	63.86	48.19	0.07790
Other Proteobacteria	1.86	0.46	0.60	0.57	0.86	2.26	1.83	0.48	5.32	2.03	ND #
Bacteroidetes	4.80 ^{ac}	11.99 ^{ac}	45.98 ^{b}	8.59 ^{ac}	30.57 ^{bc}	4.05 ^{a}	5.24 ^{ac}	1.14 ^{ac}	1.47 ^{ac}	4.49 ^{ac}	0.00018
Verrucomicrobia	7.28 ^{a}	3.66 ^{a}	8.22 ^{ac}	2.48 ^{a}	2.53 ^{a}	28.80 ^{bc}	4.87 ^{ac}	0.51 ^{a}	1.01 ^{a}	0.80 ^{a}	0.00348
Firmicutes	0.47 ^{a}	0.70 ^{a}	0.42 ^{a}	0.83 ^{a}	0.48 ^{a}	1.65 ^{a}	7.56 ^{ab}	50.13 ^{b}	22.10 ^{ab}	37.07 ^{ab}	0.00159
Planctomycetes	2.97 ^{b}	0.57 ^{ab}	0.82 ^{ab}	0.42 ^{a}	0.80 ^{ab}	2.09 ^{ab}	0.51 ^{a}	0.34 ^{a}	0.87 ^{ab}	0.50 ^{a}	0.00435
Saccharibacteria	4.87	0.41	0.13	0.07	0.22	0.06	1.02	0.02	0.25	0.09	0.13700
Other Phylum	2.17	0.42	0.54	0.28	0.39	0.18	1.02	0.49	0.56	0.39	ND #

a, b, c. Values statistically different from each other based on Tukey adjustment are distinguished by different superscripts; *determined by ANOVA; #ANOVA was not performed for these groups because they include multiple ranks of the same taxonomic level (i.e. orders or phylum).

Amongst the eight taxonomic groups described above, six were found to vary significantly across experimental groups ($P < 0.05$). Notably, Rhodobacterales were found at their highest abundance in the SIS line on day 43, i.e. after completion of the adaptation period and prior to the addition of probiotic, then decreased during the following 28 days ($P < 0.05$). In contrast, levels of Rhodobacterales did not vary significantly in the OI line. Verrucomicrobia exhibited an opposite profile, with highest levels in the OI genetic line on day 43, followed by lower abundance at later time points ($P < 0.05$). In contrast, the abundance of the members of this phylum was not found to vary significantly across samples for the SIS genetic line. While they were not supported by Tukey adjusted *P* values, other taxonomic groups exhibited abundance patterns that were suggestive of a response to treatment or trial parameters. In the OI genetic line, for instance, members of the phylum Firmicutes were found in higher abundance in samples collected on days 57 and 71 compared to day 43, with means of 22.10 – 37.07% on d71. Firmicutes in SIS shrimp guts were in contrast present in very low abundance, with means ranging from 0.42 to 0.83. Bacteroidetes in the SIS genetic line were found to increase after the day 43 time point, with abundances in probiotic treated samples found to be 3.5 -3.8 X higher than the no probiotic controls of the same time points.

4.3. Comparative Analysis of Gut Bacterial Communities by OTU Composition

A total of 2,195 OTUs were identified across all samples (Supplementary table), with 707 corresponding to previously described OTUs (Landsman, St-Pierre, Rosales-Leija, Brown, & Gibbons, 2019). OTUs that were common to both SIS and OI genetic lines represented the vast majority (99.3 – 99.7%) of sequence reads generated in the

present study (Figure 1). However, since the taxonomic analysis, as described in section 4.2, had indicated differences in composition between genetic lines, these combined results suggested at that point that SIS and OI shrimp shared common OTUs that were present at different abundance levels in their respective genetic lines. To gain further insight, Principal Coordinate Analysis (PCoA) was performed to investigate □□diversity. From this analysis, samples were found to cluster in a pattern that was indicative of differences in OTU composition between genetic lines (Figure 2A). PCoA results also suggested that changes in OTU composition had occurred in both genetic lines between the beginning and the end of the trial (Figure 2B).

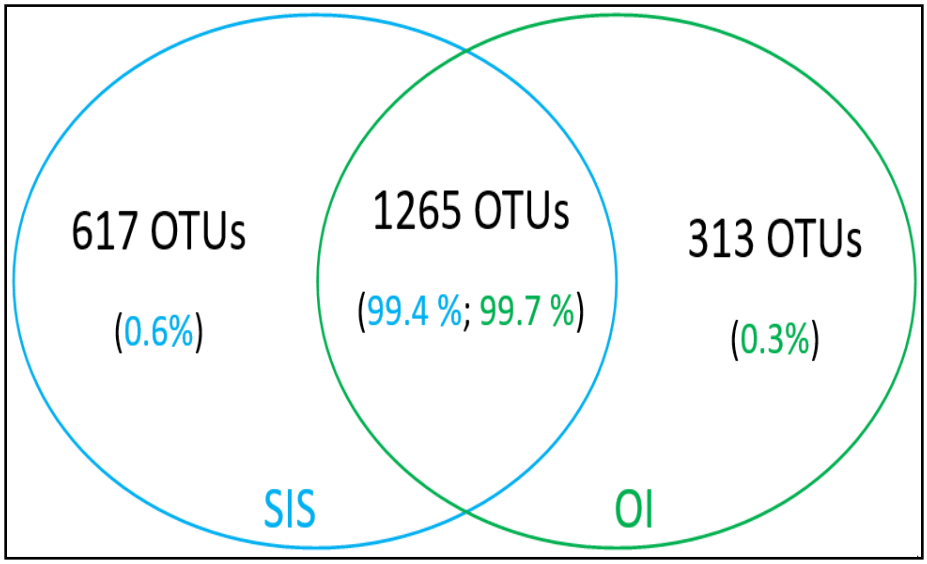

Figure 1. Venn diagram showing the number of shared and unique intestinal bacterial OTUs between the SIS and OI genetic lines of whiteleg shrimp raised in an indoor facility. Also shown is the proportion of sequence reads for each category.

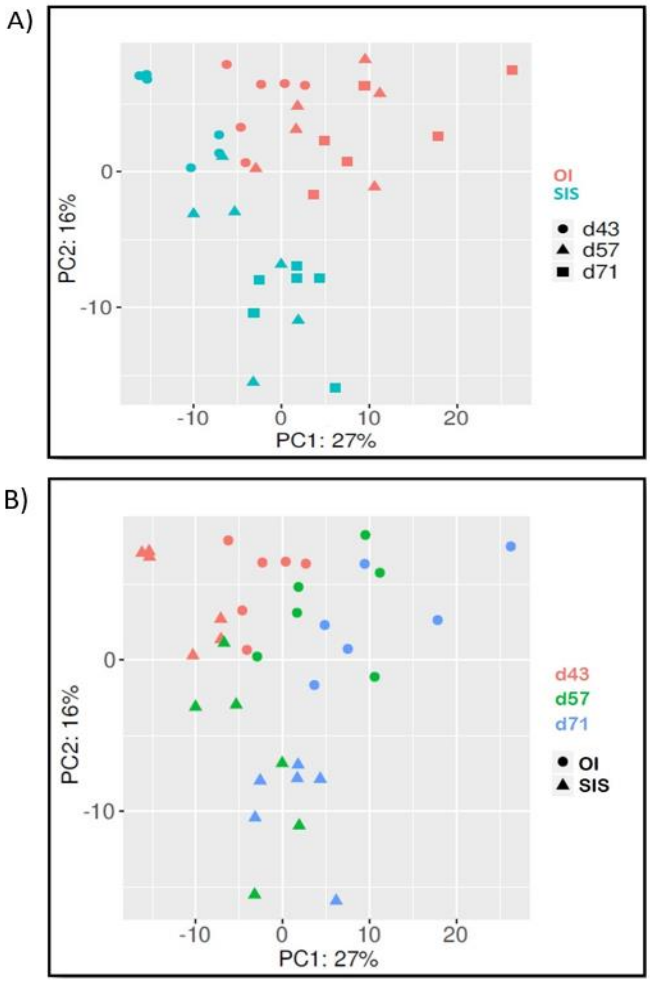

Figure 2. Comparison of intestinal bacterial communities from whiteleg shrimp using Principle Coordinate Analysis (PCoA). The x and y axes correspond to Principal Components 1 (PC1) and 2 (PC2), which explained the highest level of variation. Both panels show the same ordination graph, highlighting differences in profile either between genetic lines (A) or tenure in production tank (B).

To further investigate the changes in bacterial community composition that had occurred during the trial, the distribution profiles of the most abundant OTUs were analyzed (Table 3). Five of these major OTUs (SD_Shr-00002, SD_Shr-00003, SD_Shr-00004, SD_Shr-00006 and SD_Shr-00010) had previously been described by our group (Landsman et al., 2019), while SD_Shr-00097 (Bacteroidetes-affiliated) and SD_Shr-00098 (Verrucomicrobia-affiliated) were novel OTUs. While SD_Shr-00098 was very

closely related to *Haloferula rosea* (99.4%), the sequence identity of SD_Shr-00097 to its closest valid relative was only 93.2% (*Salinimicrobium catena*) indicating that it likely belonged to a bacterial phylogenic lineage that has yet to be described. SD_Shr-00097 was found to be almost identical (99.4%) to the 16S rRNA sequence of an uncultured bacterial species that was identified in a recirculating mariculture system (GenBank sequence JX306764).The abundance means of six of the major OTUs were found to vary significantly across experimental groups ($P < 0.05$). For Proteobacteria-affiliated main OTUs, two distinct patterns were observed. SD_Shr-00006 was in highest abundance in the SIS samples collected on d43 ($P < 0.05$), which was greater by 7.3 - 7.9X than d57 samples and by 21.9 – 24.3X than d71 samples of the same genetic line. In OI shrimp, however, the variation in abundance of SD_Shr-00006 across time points was not found to be statistically significant. SD_Shr-00004 showed an opposite profile to SD_Shr-00006, with highest abundance ($P < 0.05$) in the SIS d71 samples and lowest in the SIS d43 samples, either with probiotic supplementation (10.3X higher abundance) or without (13.9X higher abundance). SD_Shr-00097 displayed an intriguing abundance profile, with highest abundance ($P < 0.05$) in samples from SIS production tanks at d57 and d71 that were not supplemented with probiotics. This SD_Shr-00097 profile was not observed in the OI genetic line. For the Verrucomicrobia affiliated OTU SD_Shr-00098, highest abundance was observed in the d43 samples from the OI genetic line, with much lower levels in d71 samples ($P < 0.05$), by a factor of 35.1X to 49.5X. This pattern was not observed in SIS shrimp.

Table 3. Mean relative abundance (%) of main bacterial Operational Taxonomic Units in the intestinal tract of whiteleg shrimp from two genetic lines (SIS or OI), in the presence (+) or absence (-) of probiotic treatment, at three different sampling time points (d43, d57 and d71).

OTUs	SIS.43	SIS.57+	SIS.57-	SIS.71+	SIS.71-	OI.43	OI.57+	OI.57-	OI.71+	OI.71-	*P* values*
Proteobacteria											
SD_Shr-00002	6.89 ^{bc}	2.24 ^{a}	3.07 ^{ac}	0.83 ^{a}	1.91 ^{a}	1.73 ^{a}	2.48 ^{a}	0.56 ^{a}	0.59 ^{a}	0.68 ^{a}	0.00011
SD_Shr-00004	2.38 ^{a}	7.45 ^{ac}	7.22 ^{ac}	24.58 ^{bc}	33.16 ^{b}	2.09 ^{a}	3.88 ^{a}	3.03 ^{a}	6.03 ^{a}	5.63 ^{a}	6.22E-06
SD_Shr-00006	21.91 ^{b}	2.78 ^{a}	3.02 ^{a}	1.00 ^{a}	0.90 ^{a}	6.82 ^{a}	4.54 ^{a}	0.39 ^{a}	1.08 ^{a}	2.92 ^{a}	0.000824
SD_Shr-00010	22.02	58.69	21.32	50.01	14.78	40.06	49.16	38.13	48.11	34.66	0.114
Firmicutes											
SD_Shr-00003	0.39 ^{a}	0.52 ^{a}	0.33 ^{a}	0.64 ^{a}	0.38 ^{a}	1.56 ^{a}	2.81 ^{a}	49.00 ^{b}	21.44 ^{ab}	36.14 ^{ab}	0.00161
Bacteroidetes											
SD_Shr-00097	1.95 ^{a}	8.13 ^{a}	40.11 ^{bc}	2.11 ^{a}	25.68 ^{ac}	3.13 ^{a}	2.46 ^{a}	0.68 ^{a}	0.60 ^{a}	2.30 ^{a}	0.000345
Verrucomicrobia											
SD_Shr-00098	2.50 ^{a}	1.26 ^{a}	6.43 ^{ab}	0.42 ^{a}	0.83 ^{a}	25.27 ^{b}	4.18 ^{ab}	0.41 ^{a}	0.72 ^{a}	0.51 ^{a}	0.00626

a, b, c. Values statistically different from each other based on Tukey adjustment are distinguished by different superscripts; *determined by ANOVA; #ANOVA was not performed for these groups because they include multiple ranks of the same taxonomic level (i.e. orders or phylum).

5. Discussion

In the shrimp industry, there has been an increasing reliance on aquaculture to provide a steady and safe source of product. While this represents an attractive opportunity for expansion, it has also raised concerns and challenges with regards to animal health, mitigation of disease outbreaks, as well as environmental and economic sustainability. Compared to pond systems, indoor facilities have the advantage of providing better control of ambient conditions and reduced exposure to pathogens. However, since they require more costly infrastructure to operate, indoor production systems need to pay close attention to other management practices in order to remain economically competitive. A common strategy has been to use feed formulations with higher levels of plant-based products, as they are less costly than traditionally used ingredients such as fish meals and oils. However, plant-based products include a higher concentration of polysaccharides than what is typically found in diets of shrimp living in natural environments. Because most shrimp health problems are nutritionally related (E. Li et al., 2018), feeding plant ingredients could affect animal health, thus potentially reducing disease resistance in indoor shrimp. This could pose a serious problem, particularly if genetic lines that favor growth over disease resistance are used in production. In addition, minimizing exposure with natural environments may prevent gut colonization with critical symbionts that would provide long term health and / or nutritional benefits to indoor-grown shrimp, as has been described in humans and other animals.

Considering the reported importance of the gut microbiome in animal health and nutrition, the shrimp aquaculture industry would greatly benefit from developing

strategies to modulate the composition and metabolic activities of gut symbionts. However, the development of effective approaches or products requires improved insight and knowledge of the shrimp microbiome. To this end, the study presented in this report aimed at investigating the potential effects of genetic background and probiotic treatment on the composition of the developing gut bacterial communities in shrimp raised under standard indoor operating management procedures, with all production tanks provided with the same feed formulation. While there was no statistically supported differences in performance or animal health associated with genetics or probiotic treatment under the production conditions used in this study, a number of effects on the composition of gut bacterial communities in indoor grown shrimp were observed. Notably, all differences in bacterial composition were observed between the two genetic lines. For instance, while SD_Shr-00006 (Proteobacteria) and SD_Shr-00098 (Verrucomicrobia) showed a similar composition pattern, with higher abundance at d43 compared to d71 samples, their respective profiles were observed in different genetic lines. Similarly, higher abundance of SD_00097 in shrimp from probiotic supplemented tanks was observed in the SIS lines but not in the OI genetic line.

The contrast between genetic lines for the abundance of SD_Shr-00006 and SD_Shr-00098 at the d43 time point was quite striking, considering that all shrimp had been reared under the same conditions and provided with the same feed. It is thought that the initial development of the gut microbiome for shrimp is primarily acquired from artemia, a live feed that is offered shortly after hatching (E. Li et al., 2018). Bacteria phylum that dominate the shrimp gut at this stage include Proteobacteria, Bacteroidetes and Actinobacteria (E. Li et al., 2018). The microbiome then continues to develop as its

host grows, and its composition is influenced by a number of factors that include dietary ingredients and developmental stage of the host. While other parameters, such as shrimp health status as well as environmental conditions (e.g. water chemistry and water microbiology), can also affect the composition of gut microbial communities (Xiong et al., 2017), they would not be expected to have played an impactful role in this study, since no statistically supported differences for these conditions were observed.

Another surprising observation from this study was the effect of probiotics supplementation on the composition of shrimp gut bacterial communities. Indeed, SD_Shr-00097 showed a statistically supported effect, and SD_Shr-00010 showed its highest abundance in samples from probiotic supplemented tanks, although these were found to be only numerical differences. Probiotics supplementation is a common practice in indoor shrimp aquaculture as a source of microorganisms, i.e. as a means to mimic natural aquatic systems that harbor beneficial bacteria, such as the genera *Lactobacillus*, *Streptococcus*, and *Bacillus*, in sediments and water (Cornejo-Granados et al., 2017). Some studies have reported that stress can reduce growth of these bacteria and other natural microbiota, allowing uncontrolled growth of pathogenic bacteria and eventually disease (Xiong et al., 2017). As *Pediococcus*, *Lactobacillus*, and *Bacillus*-affiliated OTUs were found in only very low abundance or were undetectable in the gut of probiotic supplemented shrimp, bacterial species from the commercial probiotic formulation did not appear to efficiently colonize the shrimp gut in this study. However, their presence or absence did impact the development of gut bacterial communities. While future investigations will be necessary to uncover the mechanisms involved, it could be hypothesized that, even if probiotic bacterial species do not become established in high

density in the gut of exposed shrimp, they produce metabolites that favor the establishment of certain bacterial species or OTUs over others.

Opportunistic pathogens tend to always be present in the gut of healthy animals in low abundance. They remain benign under normal conditions (Huang et al., 2016), with disease occurring when conditions such as accumulating stress or poor diets favor their proliferation. In shrimp, the most common pathogens belong to the family Enterobacteriaceae; these include species of the genera *Pseudomonas*, *Flavobacterium*, *Escherichia*, *Aeromonas*, *Vibrio*, *Rickettsia*, *Shewanella*, and *Desulfovibrio* (E. Li et al., 2018). Of these genera, only *Vibrio* was identified in this study. Intriguingly, not only were *Vibrio*-related sequence reads very abundant across all experimental groups, their two most abundant OTUs were most closely related to *Vibrio alginolyticus* (SD_Shr-00004) and *Vibrio shilonii* (SD_Shr-00010). Both species are known to cause disease in shrimp, with *V. alginolyticus* thought to be more pathogenic by resulting in failure to thrive and ultimately death of infected hosts, while *V. shilonii* infection is more likely to cause tissue damage and reduced weight gain (Longyant et al., 2008). Interestingly, SD_Shr-00004 had previously been identified by our group as prominent in outdoor systems while found in very low abundance in indoor-aquaculture raised shrimp, which had suggested that husbandry practices may play an important role in controlling the abundance of this OTU (Landsman et al., 2019). However, SD_Shr-00004 was found in much higher abundance in this study. Intriguingly, the highest levels were observed in the SIS genetic line, which has been bred for disease resistance, and not the OI genetic line, which was bred for growth. Similarly, SD_Shr-00010 was found in high abundance in both genetic lines, with no statistically supported differences between them. These

observations suggest that these OTUs may not correspond to a pathogenic strain of their respective closest relatives.

Together, the results presented in this report show that host genetic background is an important determinant of gut bacterial composition in aquaculture-raised whiteleg shrimp. They further indicate that development of strategies to effectively manipulate the microbiome of this important seafood will need to take into consideration the genetic background of the various lines used in aquaculture production.

CONCLUSIONS

This research aimed first to identify gut microbiomes recovered from Pacific whiteleg shrimp (*Litopenaeus vannamei*) grown in various aquaculture production systems, compared to a wild population. The second goal was to determine if addition of probiotics into the aquaculture systems could beneficially modulate the gut microbiome of shrimp grown in a biosecure indoor facility. I found that there were distinctive gut microbiome profiles associated with each of the various production methods tested. Moreover, I noted that genetically distinct strains of Pacific whiteleg shrimp had unique microbiome profiles, and that these microbiomes could be altered through the use of probiotics.

In the initial experiments, pond-raised shrimp were obtained from a well-established aquaculture farm near Sanora, Mexico. The production practices, as well as source of PLs used on this farm, provided predictable, consistent performance over time, thus making it an excellent source of pond-raised shrimp. Indoor aquaculture-raised shrimp were sourced from a Midwestern facility, the trū Shrimp Company, Balaton, MN, that used a recirculating aquaculture system. Shrimp from this same facility were also used in subsequent experiments assessing the use of probiotics. Wild caught shrimp, although short in sample availability, were obtained from the ocean waters off the west coast of Mexico. This location was selected since shrimp are known to be native to the area, and thus would likely not be a result of escaped pond-raised shrimp.

I found that the gut microbiome profiles were drastically different between the pond-raised and indoor-raised shrimp, with the wild-caught shrimp sharing portions of both profiles. Bacteria affiliated with Rhodobacteraceae (Proteobacteria) and Actinobacteria were significantly more abundant in indoor-raised shrimp compared to

pond-raised shrimp (84.4% vs. 5.1%; 3.0% vs. 0.06%, respectively). Meanwhile, Vibrionaceae (Proteobacteria), Firmicutes, Fusobacteria, and Cyanobacteria were predominant in pond-raised shrimp compared to indoor-raised shrimp (44.8% vs. 0.03%; 36.0% vs 0.7%; 7.9% vs. 0.0%; 1.6% vs. 0.001%, respectively). Accordingly, the abundance of 11 of the 12 most prominent Operational Taxonomic Units (OTUs) were found to be statistically different between these two production environments. The stark difference between the gut microbiome profiles was far greater than expected and demonstrated that the production method had a large influence on gut microbiome development. Pond-raised and wild-caught shrimp were likely exposed to a great diversity of bacteria in sediment and ocean water, compared to shrimp raised in indoor tanks with reclaimed recirculating water.

Another influence on the gut microbiome is believed to include diet. While other studies have shown dependence, I did not observe any detectable difference in the indoor-raised shrimp that were fed three different diets. In contrast, all pond shrimp were fed the same diet and showed a wider microbiome diversity between sample sites, supporting the importance of production method in establishing gut microbiome profile.

The second trial was a 2 x 2 design in which one treatment was shrimp variety and the second treatment was use of a probiotic. The two genetic lines of *Litopenaeus vannamei* used were: Shrimp Improvement Systems (SIS, Islamorada, Florida, USA), selected for disease resistance, and Oceanic Institute (OI, Oahu, Hawaii, USA), selected for growth. The other treatment was a comparison of either providing a commercial probiotic supplement or not using the supplement. Probiotic treatment tanks received a daily dose of the probiotic BioWish 3P (BioWish Technologies, Cincinnati, USA) for 28

days, at a rate of 0.73 g/day. The BioWish 3P product contained *Pediococcus acidilactici* ($\geq 1 \times 10^8$ cfu/g), *Pediococcus pentosaceus* ($\geq 1 \times 10^8$ cfu/g), *Lactobacillus plantarum* ($\geq 1 \times 10^8$ cfu/g), and *Bacillus subtilis* ($\geq 1 \times 10^7$ cfu/g). The probiotic was provided with the feed but not incorporated into it, which was offered over a 24 h period. Other than the probiotic additions, all tanks were managed identically.

The results confirmed the indoor aquaculture system supported development and maintenance of a consistent and somewhat predictable gut microbiome. Shared OTUs composed a high abundance (49.7-90.7%) of the total OTUs found under all treatments applied across all trials in the indoor system. Consistent with my initial exploratory trial and previously published reports on bacterial communities of the shrimp gut, Proteobacteria was found to be the most abundant phylum in the second study, with means of experimental groups ranging between 43.68 and 80.84%. While found in lower abundance, the phyla Bacteroidetes (1.14–45.98%), Firmicutes (0.42–50.13%), and Verrucomicrobia (0.51–28.80%) were well represented across experimental groups. Other minor phyla, such as Planctomycetes (0.34–2.97%) and Saccharibacteria (0.02–4.87%), were also identified. Among the eight taxonomic groups described, six were found to vary significantly across experimental groups ($p < 0.05$).

Genetic selection played a large role in microbiome development and subsequent drift, evident over the time-lapsed samples, as it varied greatly between shrimp selected for disease resistance versus those selected for growth. In the OI shrimp genetic line, members of the phylum Firmicutes were found in higher abundance in samples collected on days 57 and 71 compared to day 43, with means of 22.10–37.07% on day 71. Firmicutes in SIS shrimp microbiome were, in contrast, present in very low abundance,

with means ranging from 0.42 to 0.83%. Bacteroidetes in the SIS genetic line were found to increase after the day 43 time point, with abundances in the absence of probiotics observed to be 3.5–3.8 times higher than in probiotic-treated shrimp at the same time points.

Interestingly, the microbial genera contained within the probiotic treatment remained almost undetectable at all sampling points, possibly due to incompatibility with the established gut microflora. The probiotics did, however, appear to induce a response in the existing gut microbiome, possibly through metabolite production. However, more research will have to be conducted to determine the actual cause and degree of effect.

In the SIS shrimp, Rhodobacterales were found at their highest abundance on day 43, after completion of the adaptation period, but prior to the addition of the probiotic. Rhodobacterales levels then decreased over the following 28 days. In contrast, levels of Rhodobacterales were more stable in the OI shrimp. Verrucomicrobia exhibited the opposite trend, with highest levels in the OI shrimp on day 43, followed by lower abundance at later time points. In contrast, the abundance of the members of this phylum were more stable in the SIS genetic line.

This project has led to a better understanding of the effectors of the gut microbiome in shrimp. These results indicate that aquaculture practices greatly influence the intestinal bacterial profile of whiteleg shrimp, and further suggest the bacterial communities of this economically important crustacean could be effectively manipulated using genetic selection and controlled environmental factors. Future research should now focus on the functional aspects of the gut microbiome and seek to understand how diet

formulation (including probiotics) can be modified to promote optimal first-pass absorption and growth.

APPENDIX

Table 1. Chapter 2; Average weight obtained from 90 randomly sampled shrimp.

Shrimp Age	SIS.ST1	SIS.ST2	SIS.ST3	SIS.STA	SIS.STB	SIS.STC	OI.ST1	OI.ST2	OI.ST3	OI.STA	OI.STB	OI.STC
43	2.261	2.710	2.604	2.657	2.992	2.657	2.406	2.270	2.364	2.678	2.199	2.331
50	3.717	4.759	3.859	3.801	4.583	3.657	3.794	3.031	3.789	4.354	3.895	4.702
57	5.206	6.286	5.557	6.484	6.308	5.941	5.598	5.114	6.516	5.836	5.130	6.427
64	7.936	9.525	8.383	8.599	9.572	7.967	8.272	7.379	8.611	8.555	8.419	8.997
71	9.508	12.269	11.124	10.640	11.630	10.460	10.243	10.066	12.045	10.451	10.318	12.173

Table 2. Chapter 2; Average daily gain obtained from subtracting previous weekly average shrimp weight from current weekly average shrimp weight and dividing by seven.

Shrimp Age	SIS.ST1	SIS.ST2	SIS.ST3	SIS.STA	SIS.STB	SIS.STC	OI.ST1	OI.ST2	OI.ST3	OI.STA	OI.STB	OI.STC
50	0.208	0.292	0.179	0.163	0.227	0.142	0.198	0.108	0.203	0.239	0.242	0.338
57	0.212	0.218	0.242	0.383	0.246	0.326	0.257	0.297	0.389	0.211	0.176	0.246
64	0.390	0.462	0.403	0.302	0.441	0.289	0.381	0.323	0.299	0.388	0.469	0.367
71	0.223	0.331	0.391	0.298	0.265	0.382	0.281	0.383	0.490	0.270	0.271	0.453

Table 3. Chapter 2; Stocking, stocking density, termination count, termination density, mortality rate and daily mortality rate data.

	SIS.ST1	SIS.ST2	SIS.ST3	SIS.STA	SIS.STB	SIS.STC	OI.ST1	OI.ST2	OI.ST3	OI.STA	OI.STB	OI.STC
STOCK	1999	2002	2006	2006	2000	2003	1943	1943	1944	1943	1945	1945
S.DENSITY	0.16	0.181	0.188	0.161	0.181	0.188	0.202	0.202	0.202	0.202	0.202	0.202
TERM	1265	1033	1436	1229	1193	1251	1287	1229	1175	1191	1164	1055
T.DENSITY	1.437	1.675	1.481	1.535	1.667	1.431	1.541	1.518	1.782	1.57	1.566	1.764
MORT	16.7%	36.6%	22.5%	26.5%	28.0%	24.1%	19.1%	22.9%	25.3%	24.8%	26.2%	30.1%
DMORT	0.39%	0.85%	0.52%	0.62%	0.65%	0.56%	0.45%	0.53%	0.59%	0.58%	0.61%	0.70%

Table 4. Chapter 2; Daily total ammonia nitrogen results from Hach spectrophotometer analysis.

Shrimp Age	SIS.ST1	SIS.ST2	SIS.ST3	SIS.STA	SIS.STB	SIS.STC	OI.ST1	OI.ST2	OI.ST3	OI.STA	OI.STB	OI.STC
42	0.21	0.24	0.17	0.22	0.18	0.20	0.33	0.63	0.74	0.31	2.40	2.84
43	0.20	0.26	0.16	0.24	0.27	0.25	1.80	1.38	1.60	0.12	1.00	0.84
44	0.22	0.22	0.24	0.29	0.17	0.35	0.38	0.88	0.72	0.35	1.36	1.45
45	0.22	0.23	0.23	0.19	0.29	0.20	0.25	0.92	0.62	0.26	0.72	0.98
46	0.28	0.32	0.30	0.24	0.30	0.38	0.52	0.78	0.84	0.18	0.66	0.78
47	0.26	0.25	0.22	0.25	0.29	0.33	0.26	0.61	0.80	0.09	0.50	0.56
48	0.22	0.23	0.22	0.21	0.23	0.24	0.18	0.62	0.80	0.05	0.24	0.42
49	0.21	0.20	0.25	0.19	0.24	0.32	0.12	0.65	0.30	0.13	0.59	0.16
50	0.28	0.15	0.24	0.27	0.25	0.31	0.11	0.22	0.37	0.10	0.45	0.38
51	0.24	0.24	0.22	0.24	0.25	0.31	0.10	0.12	0.07	0.03	0.09	0.08
52	0.15	0.29	0.33	0.21	0.37	0.33	0.22	0.25	0.18	0.15	0.47	0.52
53	0.17	0.29	0.32	0.22	0.41	0.38	0.21	0.10	0.13	0.23	0.19	0.10
54	0.20	0.29	0.31	0.24	0.46	0.42	0.26	0.22	0.31	0.24	0.30	0.38
55	0.32	0.23	0.28	0.15	0.40	0.42	0.37	0.37	0.35	0.32	0.25	0.26
56	0.19	0.28	0.24	0.14	0.24	0.29	0.36	0.31	0.28	0.27	0.16	0.43
57	0.21	0.15	0.25	0.24	0.27	0.17	0.41	0.32	0.32	0.32	0.44	0.36
58	0.28	0.29	0.26	0.22	0.23	0.33	0.33	0.33	0.34	0.31	0.21	0.17
59	0.26	0.31	0.26	0.29	0.30	0.24	0.37	0.34	0.32	0.35	0.47	0.36
60	0.27	0.31	0.33	0.31	0.31	0.33	0.27	0.31	0.35	0.40	0.46	0.40
61	0.25	0.29	0.30	0.24	0.30	0.29	0.34	0.27	0.27	0.37	0.39	0.31
62	0.23	0.27	0.26	0.18	0.28	0.25	0.21	0.30	0.19	0.30	0.31	0.21
63	0.11	0.12	0.18	0.10	0.18	0.08	0.09	0.28	0.33	0.18	0.31	0.29
64	0.16	0.17	0.24	0.21	0.19	0.18	0.35	0.29	0.37	0.34	0.43	0.58
65	0.26	0.23	0.28	0.27	0.35	0.34	0.46	0.28	0.35	0.43	0.43	0.62
66	0.16	0.15	0.19	0.15	0.22	0.14	0.50	0.36	0.38	0.48	0.58	0.52
67	0.28	0.32	0.32	0.27	0.52	0.31	0.29	0.22	0.32	0.28	0.34	0.31
68	0.34	0.36	0.40	0.42	0.39	0.32	0.42	0.30	0.26	0.46	0.46	0.50
69	0.32	0.26	0.31	0.32	0.42	0.37	0.36	0.38	0.38	0.48	0.70	0.52
70	0.22	0.33	0.38	0.25	0.36	0.29	0.30	0.20	0.25	0.30	0.40	0.35
71	0.42	0.22	0.52	0.24	0.29	0.26	0.36	0.28	0.28	0.41	0.36	0.24
72	0.36	0.43	0.66	0.35	0.39	0.21	0.08	0.08	0.08	0.11	0.08	0.13

Table 5. Chapter 2; Monday, Wednesday, Friday nitrite nitrogen results from Hach spectrophotometer analysis.

Shrimp Age	SIS.ST1	SIS.ST2	SIS.ST3	SIS.STA	SIS.STB	SIS.STC	OI.ST1	OI.ST2	OI.ST3	OI.STA	OI.STB	OI.STC
42	0.14	0.20	0.16	0.11	0.19	0.31	3.31	3.86	2.73	0.25	3.92	3.01
45	0.15	0.15	0.15	0.16	0.25	0.25	2.60	4.11	2.00	0.25	4.96	3.96
48	0.13	0.14	0.18	0.14	0.21	0.24	0.88	1.45	2.01	0.22	3.95	4.60
51	0.20	0.16	0.27	0.18	0.23	0.32	0.58	0.64	0.42	0.18	3.96	3.89
54	0.21	0.18	0.33	0.18	0.28	0.33	0.63	0.51	0.51	0.23	4.05	4.65
57	0.19	0.22	0.30	0.21	0.42	0.44	0.75	0.41	0.47	0.28	0.90	2.01
60	0.22	0.29	0.30	0.25	0.41	0.49	0.62	0.43	0.36	0.28	1.08	1.63
63	0.22	0.35	0.40	0.19	0.55	0.66	0.93	0.19	0.20	0.14	0.68	0.68
66	0.37	0.47	0.45	0.26	0.52	0.60	0.80	0.47	0.40	0.36	1.96	1.95
69	0.38	0.46	0.51	0.35	0.60	0.71	0.71	0.55	0.36	0.32	3.84	1.60
72	0.40	0.59	0.51	0.30	0.61	0.56	0.77	0.65	0.44	0.48	2.71	0.99

Table 6. Chapter 2; Monday, Wednesday, Friday nitrate nitrogen results from Hach spectrophotometer analysis.

Shrimp Age	SIS.ST1	SIS.ST2	SIS.ST3	SIS.STA	SIS.STB	SIS.STC	OI.ST1	OI.ST2	OI.ST3	OI.STA	OI.STB	OI.STC
42	6.55	6.20	6.37	8.58	6.90	6.02	6.28	5.14	5.58	6.55	7.79	4.49
45	8.38	8.14	8.14	4.96	10.03	7.91	6.81	6.28	6.99	6.55	6.23	4.38
48	5.08	7.67	7.25	10.74	11.44	12.98	7.08	7.67	4.34	5.84	5.80	6.85
51	13.21	7.79	13.68	12.74	11.13	10.50	9.44	6.72	7.67	7.61	6.38	7.08
54	13.80	11.80	15.81	14.86	14.98	15.10	15.57	11.56	12.74	10.15	16.87	15.57
57	8.14	7.32	12.74	11.75	14.57	11.44	7.52	6.73	10.85	7.61	14.47	6.90
60	8.05	10.85	7.87	13.21	15.45	13.96	9.11	15.59	11.05	11.93	4.60	3.84
63	12.98	9.37	12.98	11.57	16.28	12.50	10.38	8.22	16.63	11.92	12.03	10.97
66	7.34	8.05	12.15	15.92	20.29	16.39	14.16	12.63	19.34	26.06	13.69	17.22
69	15.92	16.99	22.53	19.58	20.76	23.23	16.16	20.40	22.76	20.17	22.41	13.09
72	19.35	10.86	14.61	17.23	24.90	17.23	9.71	11.43	14.29	16.41	13.47	12.74

Table 7. Chapter 2; Daily alkalinity results from Hach spectrophotometer analysis.

Shrimp Age	SIS.ST1	SIS.ST2	SIS.ST3	SIS.STA	SIS.STB	SIS.STC	OI.ST1	OI.ST2	OI.ST3	OI.STA	OI.STB	OI.STC
42	200	197	200	209	191	198	205	213	195	177	234	224
43	189	204	182	199	193	186	158	220	211	174	188	182
44	197	206	188	200	190	194	213	202	200	172	216	224
45	190	189	191	197	195	198	200	194	195	183	228	207
46	197	205	188	203	203	192	188	196	197	182	207	209
47	193	192	195	191	194	194	170	160	160	160	170	160
48	195	198	188	196	188	196	207	203	195	188	210	214
49	204	202	194	189	194	200	294	227	233	196	205	202
50	190	203	198	186	210	181	200	204	184	192	183	195
51	186	176	201	174	207	175	219	210	206	186	187	198
52	185	194	180	181	205	184	197	207	207	200	179	180
53	180	200	200	200	200	200	179	204	192	202	180	191
54	190	199	184	182	216	196	172	186	177	204	165	177
55	199	201	182	190	219	195	194	174	183	214	177	186
56	194	215	190	193	233	203	188	198	199	225	173	195
57	201	201	190	193	218	205	177	198	203	209	174	199
58	191	208	169	188	237	204	169	179	182	204	160	156
59	190	199	183	188	240	199	168	174	189	210	175	171
60	184	207	168	197	251	194	183	186	175	212	177	181
61	180	200	160	200	200	200	169	179	168	211	172	179
62	167	179	181	178	236	195	170	177	185	206	172	188
63	196	190	185	189	251	219	187	195	210	228	188	208
64	182	168	173	177	256	203	182	192	195	218	179	213
65	179	179	187	181	232	201	177	183	195	222	176	191
66	179	182	173	178	243	197	184	178	187	209	180	208
67	181	168	185	179	256	212	173	187	186	199	172	193
68	185	200	201	195	266	220	179	180	179	195	195	201
69	182	189	189	185	258	216	169	179	202	200	186	197
70	188	187	207	198	284	212	177	173	185	184	159	176
71	165	169	196	200	271	225	183	182	190	193	190	211
72	189	177	188	201	275	225	189	215	205	208	193	208

Table 8. Chapter 2; Daily pH results from hand-held YSI meter.

Shrimp Age	SIS.ST1	SIS.ST2	SIS.ST3	SIS.STA	SIS.STB	SIS.STC	OI.ST1	OI.ST2	OI.ST3	OI.STA	OI.STB	OI.STC
42	7.56	7.56	7.62	7.67	7.57	7.59	7.93	7.74	7.77	7.65	7.90	7.70
43	7.46	7.41	7.60	7.58	7.45	7.54	7.81	7.76	7.76	7.76	7.89	7.89
44	7.50	7.54	7.69	7.66	7.47	7.59	7.86	7.79	7.81	7.65	7.86	7.77
45	7.35	7.38	7.49	7.46	7.32	7.53	7.79	7.67	7.71	7.61	7.81	7.72
46	7.45	7.42	7.50	7.48	7.35	7.45	7.80	7.67	7.73	7.57	7.81	7.69
47	7.44	7.41	7.46	7.41	7.42	7.43	7.23	7.24	7.31	7.18	7.35	7.32
48	7.75	7.62	7.64	7.62	7.54	7.55	7.77	7.67	7.74	7.61	7.77	7.64
49	7.33	7.36	7.40	7.44	7.38	7.40	7.58	7.65	7.73	7.59	7.72	7.70
50	7.59	7.50	7.52	7.52	7.30	7.45	7.64	7.57	7.63	7.51	7.61	7.58
51	7.49	7.52	7.55	7.54	7.51	7.51	7.51	7.52	7.59	7.27	7.59	7.50
52	7.44	7.36	7.37	7.33	7.25	7.37	7.54	7.50	7.52	7.30	7.55	7.42
53	7.25	7.26	7.36	7.40	7.28	7.33	7.55	7.62	7.61	7.36	7.49	7.40
54	7.37	7.42	7.47	7.46	7.31	7.31	7.37	7.37	7.35	7.29	7.42	7.35
55	7.41	7.35	7.45	7.36	7.22	7.33	7.43	7.39	7.29	7.30	7.40	7.25
56	7.34	7.39	7.43	7.39	7.32	7.38	7.42	7.44	7.29	7.21	7.46	7.38
57	7.55	7.53	7.45	7.40	7.36	7.47	7.50	7.59	7.59	7.40	7.52	7.42
58	7.50	7.48	7.44	7.38	7.33	7.40	7.46	7.39	7.49	7.24	7.78	7.78
59	7.43	7.45	7.46	7.40	7.32	7.39	7.38	7.35	7.40	7.30	7.31	7.31
60	7.40	7.41	7.40	7.35	7.33	7.36	7.40	7.34	7.24	7.25	7.34	7.28
61	7.34	7.41	7.36	7.39	7.35	7.34	7.44	7.42	7.32	7.31	7.40	7.37
62	7.38	7.44	7.48	7.46	7.41	7.44	7.52	7.51	7.45	7.40	7.47	7.40
63	7.30	7.37	7.40	7.41	7.34	7.37	7.46	7.38	7.40	7.36	7.39	7.34
64	7.54	7.51	7.50	7.35	7.35	7.41	7.25	7.30	7.20	7.29	7.37	7.27
65	7.48	7.43	7.40	7.48	7.27	7.34	7.34	7.38	7.36	7.29	7.35	7.33
66	7.33	7.40	7.37	7.34	7.38	7.34	7.48	7.50	7.53	7.44	7.54	7.43
67	7.39	7.35	7.37	7.34	7.24	7.29	7.37	7.38	7.37	7.31	7.34	7.28
68	7.34	7.35	7.36	7.32	7.24	7.34	7.46	7.47	7.38	7.34	7.42	7.29
69	7.33	7.32	7.34	7.30	7.21	7.31	7.41	7.35	7.41	7.33	7.42	7.21
70	7.43	7.45	7.44	7.41	7.41	7.39	7.41	7.38	7.39	7.28	7.32	7.24
71	7.33	7.39	7.35	7.31	7.34	7.35	7.30	7.14	7.24	7.18	7.38	7.50
72	7.27	7.29	7.34	7.35	7.40	7.30	7.34	7.42	7.21	7.31	7.39	7.29

Table 9. Chapter 2; Weekly inert vibrio colony forming unit plate results from independent lab RTI.

Shrimp Age	SIS.ST1	SIS.ST2	SIS.ST3	SIS.STA	SIS.STB	SIS.STC	OI.ST1	OI.ST2	OI.ST3	OI.STA	OI.STB	OI.STC
39	1300	590	370	1200	380	220	8300	3800	920	3200	2200	1100
46	2800	1400	2300	3000	2600	2800	5460	4740	4020	2400	4860	11400
53	8000	1500	1600	7100	2300	26000	8800	2300	4100	7000	3600	3900
60	7100	2100	9200	6100	2800	2100	2300	800	2400	2600	15000	3800
67	11000	7000	11000	8100	6100	7500	1800	4600	7200	2600	15000	3400

Table 10. Chapter 2; Weekly total heterotrophic colony forming unit plate results from independent lab RTI.

Shrimp Age	SIS.ST1	SIS.ST2	SIS.ST3	SIS.STA	SIS.STB	SIS.STC	OI.ST1	OI.ST2	OI.ST3	OI.STA	OI.STB	OI.STC
39	410000	300000	110000	170000	180000	140000	200000	69000	190000	200000	470000	1000000
46	290000	270000	280000	190000	160000	350000	159000	117000	107000	20000	310000	370000
53	120000	140000	610000	250000	390000	660000	160000	50000	160000	30000	290000	130000
60	44000	490000	69000	61000	75000	27000	46000	40000	65000	72000	16000	130000
67	140000	230000	140000	630000	290000	270000	48000	63000	51000	58000	190000	92000

Table 11. Chapter 2; Weekly Pediococcus colony forming unit plate results from independent lab RTI.

Shrimp Age	SIS.ST1	SIS.ST2	SIS.ST3	SIS.STA	SIS.STB	SIS.STC	OI.ST1	OI.ST2	OI.ST3	OI.STA	OI.STB	OI.STC
46	100	100	0	0	100	0	0	100	100	0	600	400
53	300	0	0	0	4800	0	3000	800	0	1200	0	500
60	0	100	0	0	0	0	400	200	5000	0	0	200
67	0	5600	0	0	0	0	300	0	0	0	0	0

Table 12. Chapter 2; Weekly animal health exam results and calculations, SIS genetics.

SIS.ST1	**D43**	**D50**	**D57**	**D64**	**D71**
Weight (g)	1.972	3.614	4.654	8.570	10.618
Length (cm)	5.560	6.620	7.520	9.200	9.240
Hepatopancreas weight (g)	0.107	0.222	0.248	0.273	0.477
Antenna Length (cm)	6.710	4.270	4.550	4.440	3.590
MCF	1.147	1.246	1.094	1.101	1.346
HSI	0.054	0.061	0.053	0.032	0.045
Shrimp-Antenna Length Ratio	1.207	0.645	0.605	0.483	0.389
SIS.ST2	**D43**	**D50**	**D57**	**D64**	**D71**
Weight (g)	3.324	3.430	6.922	8.870	10.682
Length (cm)	6.800	6.400	8.500	9.300	9.840
Hepatopancreas weight (g)	0.165	0.204	0.399	0.439	0.487
Antenna Length (cm)	7.830	4.180	6.270	4.770	5.610
MCF	1.057	1.308	1.127	1.103	1.121
HSI	0.050	0.059	0.058	0.049	0.046
Shrimp-Antenna Length Ratio	1.151	0.653	0.738	0.513	0.570
SIS.ST3	**D43**	**D50**	**D57**	**D64**	**D71**
Weight (g)	2.846	3.886	5.512	6.854	11.490
Length (cm)	6.080	6.900	7.860	8.720	9.760
Hepatopancreas weight (g)	0.166	0.202	0.285	0.362	0.607
Antenna Length (cm)	7.440	7.070	5.940	5.240	6.400
MCF	1.266	1.183	1.135	1.034	1.236
HSI	0.058	0.052	0.052	0.053	0.053
Shrimp-Antenna Length Ratio	1.224	1.025	0.756	0.601	0.656
SIS.STA	**D43**	**D50**	**D57**	**D64**	**D71**
Weight (g)	3.168	4.210	6.962	8.406	11.358
Length (cm)	6.520	6.900	8.040	9.380	10.200
Hepatopancreas weight (g)	0.161	0.268	0.370	0.422	0.500
Antenna Length (cm)	8.820	6.530	5.020	4.620	5.440
MCF	1.143	1.282	1.340	1.019	1.070
HSI	0.051	0.064	0.053	0.050	0.044
Shrimp-Antenna Length Ratio	1.353	0.946	0.624	0.493	0.533
SIS.STB	**D43**	**D50**	**D57**	**D64**	**D71**
Weight (g)	4.138	5.008	6.860	10.174	11.862
Length (cm)	7.320	7.260	7.800	9.880	10.180
Hepatopancreas weight (g)	0.188	0.277	0.316	0.474	0.540
Antenna Length (cm)	8.950	6.590	7.310	5.020	6.050
MCF	1.055	1.309	1.446	1.055	1.124
HSI	0.045	0.055	0.046	0.047	0.046
Shrimp-Antenna Length Ratio	1.223	0.908	0.937	0.508	0.594
SIS.STC	**D43**	**D50**	**D57**	**D64**	**D71**
Weight (g)	2.866	3.798	6.438	7.310	10.500
Length (cm)	6.400	6.580	7.780	8.880	9.740
Hepatopancreas weight (g)	0.095	0.244	0.323	0.353	0.547
Antenna Length (cm)	6.680	7.350	4.810	4.860	6.240
MCF	1.093	1.333	1.367	1.044	1.136
HSI	0.033	0.064	0.050	0.048	0.052
Shrimp-Antenna Length Ratio	1.044	1.117	0.618	0.547	0.641

Table 13. Chapter 2; Weekly animal health exam results and calculations, OI genetics.

OI.ST1	**D43**	**D50**	**D57**	**D64**	**D71**
Weight (g)	2.326	4.744	5.386	6.672	10.668
Length (cm)	5.740	7.580	7.580	8.480	9.960
Hepatopancreas weight (g)	0.155	0.300	0.315	0.374	0.568
Antenna Length (cm)	6.830	6.760	6.040	3.540	4.740
MCF	1.230	1.089	1.237	1.094	1.080
HSI	0.067	0.063	0.059	0.056	0.053
Shrimp-Antenna Length Ratio	1.190	0.892	0.797	0.417	0.476
OI.ST2	**D43**	**D50**	**D57**	**D64**	**D71**
Weight (g)	2.212	3.160	5.092	6.726	10.142
Length (cm)	5.920	6.560	7.380	8.580	9.680
Hepatopancreas weight (g)	0.132	0.192	0.311	0.392	0.592
Antenna Length (cm)	5.270	4.920	4.390	5.530	3.370
MCF	1.066	1.119	1.267	1.065	1.118
HSI	0.060	0.061	0.061	0.058	0.058
Shrimp-Antenna Length Ratio	0.890	0.750	0.595	0.645	0.348
OI.ST3	**D43**	**D50**	**D57**	**D64**	**D71**
Weight (g)	2.584	3.842	6.636	9.884	10.324
Length (cm)	6.120	6.780	8.400	9.800	9.560
Hepatopancreas weight (g)	0.154	0.225	0.421	0.525	0.507
Antenna Length (cm)	6.120	6.750	6.030	4.680	4.460
MCF	1.127	1.233	1.120	1.050	1.182
HSI	0.060	0.059	0.064	0.053	0.049
Shrimp-Antenna Length Ratio	1.000	0.996	0.718	0.478	0.467
OI.STA	**D43**	**D50**	**D57**	**D64**	**D71**
Weight (g)	3.110	4.728	5.532	8.240	10.004
Length (cm)	6.100	7.580	7.940	9.040	9.580
Hepatopancreas weight (g)	0.186	0.274	0.290	0.418	0.532
Antenna Length (cm)	5.990	4.110	3.310	5.430	4.170
MCF	1.370	1.086	1.105	1.115	1.138
HSI	0.060	0.058	0.052	0.051	0.053
Shrimp-Antenna Length Ratio	0.982	0.542	0.417	0.601	0.435
OI.STB	**D43**	**D50**	**D57**	**D64**	**D71**
Weight (g)	2.034	2.808	4.686	7.424	11.250
Length (cm)	5.520	6.260	7.540	8.820	9.860
Hepatopancreas weight (g)	0.179	0.175	0.303	0.402	0.539
Antenna Length (cm)	6.390	5.380	4.220	4.410	4.940
MCF	1.209	1.145	1.093	1.082	1.174
HSI	0.088	0.062	0.065	0.054	0.048
Shrimp-Antenna Length Ratio	1.158	0.859	0.560	0.500	0.501
OI.STC	**D43**	**D50**	**D57**	**D64**	**D71**
Weight (g)	2.498	3.352	7.482	10.022	13.706
Length (cm)	5.860	6.560	8.900	9.680	10.540
Hepatopancreas weight (g)	0.156	0.200	0.426	0.504	0.682
Antenna Length (cm)	6.150	6.760	6.410	3.980	6.290
MCF	1.241	1.187	1.061	1.105	1.171
HSI	0.062	0.060	0.057	0.050	0.050
Shrimp-Antenna Length Ratio	1.049	1.030	0.720	0.411	0.597

Table 14. Chapter 2; Daily feed offered.

Shrimp Age	SIS.ST1	SIS.ST2	SIS.ST3	SIS.STA	SIS.STB	SIS.STC	OI.ST1	OI.ST2	OI.ST3	OI.STA	OI.STB	OI.STC
44	246	246	246	246	246	246	244	244	246	243	244	244
45	259	259	259	260	259	259	258	258	258	256	258	258
46	273	273	273	274	273	273	271	271	272	270	271	271
47	287	288	288	288	287	288	286	286	286	285	286	286
48	303	303	304	304	303	303	301	301	302	300	302	302
49	319	320	320	320	319	320	317	317	318	316	318	318
50	326	327	325	327	326	325	325	325	326	324	326	326
51	335	336	334	336	335	334	329	330	331	329	331	330
52	344	345	343	345	344	343	338	339	340	338	340	339
53	353	354	352	354	353	352	347	348	349	347	349	348
54	362	362	361	363	362	360	355	356	357	355	357	356
55	370	371	369	371	370	368	363	364	365	363	366	364
56	378	379	377	379	378	376	371	372	373	371	374	372
57	386	387	385	387	386	384	379	380	381	379	381	380
58	385	386	383	383	382	380	381	382	383	379	383	379
59	392	393	390	390	389	388	388	389	390	387	391	387
60	399	400	397	397	396	394	395	396	397	394	398	393
61	406	407	404	404	403	401	402	403	404	400	404	400
62	413	414	411	411	410	408	408	409	411	407	411	407
63	419	420	417	417	416	414	415	416	417	413	418	413
64	426	426	424	423	422	420	421	422	423	420	424	419
65	423	426	423	424	421	421	424	424	420	424	428	423
66	429	432	429	430	427	427	430	430	426	430	434	429
67	435	438	434	435	433	432	436	436	432	435	439	435
68	436	439	436	437	434	434	437	438	433	437	441	436
69	433	436	433	434	431	431	434	435	430	434	438	433
70	430	434	430	431	428	428	431	432	428	431	435	430
71	428	431	427	428	426	425	429	429	425	428	432	428

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